Université de Montréal

Morphological and functional characterization of placenta during gestation in bovine clones derived by somatic nuclear transfer

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RÉSUMÉ

La technique de clonage par transfert nucléaire de cellules somatiques (SCNT) présente une page importante dans les annales scientifiques, mais son application pratique demeure incertaine dû à son faible taux de succès. Les anomalies placentaires et de développement fœtal se traduisent par des pertes importantes de gestation et des mortalités néonatales.

Dans un premier temps, la présente étude a caractérisé les changements morphologiques des membranes fœtales durant la gestation clonée en les comparant à des gestations contrôles obtenues à partir de l'insémination artificielle. Les différentes anomalies morphologiques des placentomes telles que l'œdème chorioallantoique, la présence de zones hyperéchoiques et irrégulières dans la membrane amniotique et la présence de cellules inflammatoires dégénérées compromettent le développement fœtal normal de la gestation clonée. L'examen ultrasonographique représente une technique diagnostique importante pour faire le suivi d'une gestation et de caractériser les changements placentaires dans le cadre d'évaluation globale du bien-être fœtal.

Le profil hormonal de trois stéroïdes (progestérone (P4), estrone sulfate (E1S), et œstradiol (E2)) et de la protéine B spécifique de gestation (PSPB) dans le sérum des vaches porteuses de clones SCNT a été déterminé et associé aux anomalies de gestations clonées. Une diminution de la P4 sérique au jour 80, une élévation du niveau de la concentration de la PSPB au jour 150, et une augmentation de la concentration d'E2 sérique durant le

deuxième et troisième tiers de la gestation clonée coïncident avec les anomalies de gestation déjà reportées. Ces changements du profil hormonal associés aux anomalies phénotypiques du placenta compromettent le déroulement normal de la gestation clonée et gênent le développement et le bien-être fœtal.

Sur la base des observations faites sur le placenta de gestation clonée, le mécanisme moléculaire pouvant expliquer la disparition de l'épithélium du placenta (l'interface entre le tissue maternel et le placenta) a été étudié. L'étude a identifié des changements dans l'expression de deux protéines d'adhérence (E-cadhérin et β-catenin) de cellules épithéliales pouvant être associées aux anomalies du placenta chez les gestations clonées. Le tissu de cotylédons provenant de gestations clonées et contrôles a été analysé par Western blot, RT-PCR quantitatif, et par immunohistochimie. Les résultats présentaient une diminution significative ($p<0.05$) de l'expression des dites protéines dans les cellules trophoblastiques chez les gestations clonées. Le RT-PCR quantitatif démontrait que les gènes CCND1, CLDN1 et MSX1 ciblés par la voie de signalisation de la Wnt/β-catenin étaient significativement sous exprimés. La diminution de l'expression des protéines E-cadherin et β-catenin avec une réduction de l'activation de la protéine β-catenin durant le période d'attachement de l'embryon peut potentiellement expliquer l'absence totale ou partielle de l'attachement des membranes fœtales au tissu maternel et éventuellement, l'insuffisance placentaire caractéristique des gestations clonées chez la vache.

La caractérisation morphologique et fonctionnelle du placenta durant les gestations clonées à haut risque est essentielle pour évaluer le statut de la gestation. Les résultats de la présente étude permettront de prédire le développement et le bien-être fœtal de façon critique à travers un protocole standardisé et permettre des interventions médicales pour améliorer le taux de succès des gestations clonées chez les bovins.

Mots-clés : Bovin, Suivi des gestations, Clone, Profil hormonal, Molécules d'adhésion

ABSTRACT

Although somatic cell nuclear transfer (SCNT) has been shown to be successful, there are still problems with this technique that is inhibiting its use in industry. Altered placental formation and development results in the high incidence of pregnancy loss and prenatal morbidity and mortality found in SCNT pregnancies.

The objective of the initial study was to characterize morphological changes in fetal membranes of a group of bovine recipients carrying cloned fetuses and comparing them with control AI fetuses at various stages of gestation. Several morphological anomalies of placentomes such as edematous chorioallantoic membrane, hyper-echodense spikes or irregularities in the amniotic membrane as well as the pathological presence of degenerated inflammatory cells accompanied by disappearance of the placental epithelium were observed. It was concluded that these anomalies compromise fetal development. The results of this work also showed that ultrasonography may be a reliable technique to monitor and to characterize the placental changes in bovine pregnancies that can be used to assess fetal well-being.

The next study compared the functionality of the placenta of cloned fetuses with controls. The concentration of three steroids (progesterone (P4), estrone sulphate (E1S), and estradiol (E2)) and pregnancy-specific protein B (PSPB) in maternal peripheral circulation were assessed and their associations with gestational anomalies were determined. The hormones profiles in the SCNT recipients deviated from the control group

at certain stages of pregnancy. We observed higher concentrations of E2 throughout the study period, lower levels of P4 at day 80 as well as elevated PSPB concentrations at day 150 in SCNT recipients which coincided with high rate of abortion in these animals shortly after this stage. So, it is proposed that these hormonal changes together with the morphological anomalies of the placenta result in compromised fetal development.

Finally, the molecular mechanism that could be responsible for the abnormal disappearance of the epithelial layer observed in SCNT placenta was investigated. To do so, we measured the expression of two major epithelial adherens junction proteins (E-cadherin and β-catenin) and determined if their expression is altered in relation to the aberrant placentation in SCNT embryos. Cotyledonary tissues from SCNT gestations and control pregnancies were analyzed by Western blot, quantitative RT-PCR and immunohistochemical analysis. Both candidates were significantly ($P < 0.05$) under-expressed in SCNT trophoblast cells at the protein level. Also, qRT-PCR confirmed that the Wnt/β-catenin signaling pathway target genes *CCND1, CLDN1* and *MSX1* were significantly down-regulated in SCNT placentas. So, we inferred that impaired E-cadherin and β-catenin protein expression, along with defective β-catenin signaling during embryo attachment, specifically in the window of placentation, results in loose attachment and contributes to insufficient placentation in bovine SCNT-derived embryos.

Overall, we concluded that during the high-risk pregnancy of cloned fetuses, characterization of the morphological and functional changes of the placenta is critical to

enable us to predict normal fetal development and wellbeing through a standardized procedure during clone gestations and to intervene medically in emergency cases to improve the overall efficiency of cloning in cattle.

Keywords: Bovine, Pregnancy monitoring, Cloned, Hormone profile, Adhesion molecules

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List of Abbreviations

3βHSD	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase
AI	Artificial insemination
ART	Assisted reproduction techniques
bHLH	Basic helix-loop-helix
BNC	Binucleate trophoblast cell
bPAG	Bovine pregnancy-associated glycoprotein
bPL	Bovine prolactin-related protein
BSA	Bovine serum albumen
CCND1	Cyclin D1
CDH1	cadherin 1, E-cadherin (epithelial)
CDH2	cadherin 2, N-cadherin (neuronal)
CDX2	Caudal type homeobox 2
CK1α	Casein kinase Iα
CKII	Casein kinase II
CL	Corpus luteum
CLDN1	Claudin 1
COC	Cumulus oocyte complexe
CTNNB1	Catenin (cadherin-associated protein), beta 1
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
E1S	Estrone Sulphate
E2	Estradiol
EC	Extracellular
ET	Embryo transfer

FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
FZD5	Frizzled homolog 5
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
GSK3-β	Glycogen synthase kinase 3- β
H2AFZ	H2A histone family, member Z
HES	Haematoxylin-eosin-saffran
HRP	Horseradish peroxidase
I.M.	Intra-muscular
ICM	Inner cell mass
IFNt	Interferon-tau
IGF-1	Insulin-like growth factor 1
IVP	In vitro produced
LEF1	Lymphoid enhancer factor 1
LH	Luteinizing hormone
LOS	Large offspring syndrome
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
LSM	Least squares mean
MDCK	Madin-Darby Canine Kidney
MNC	Mononucleate trophoblast cell
MSX1	Msh homeobox 1
NT	Nuclear transfer
P4	Progesterone
P450scc	Cytochrome P450, family 11, subfamily A, polypeptide 1
PAF	Paraformaldehyde

PAS	periodic acid-Schiff
PBS	Phosphate buffered saline
PPIA	Peptidylprolyl isomerase A (cyclophilin A)
PSPB	Pregnancy-specific protein B
PSTT	Placental site trophoblastic tumour
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative reverse transcriptase PCR
SCNT	Somatic cell nuclear transfer
SEM	Standard error of the mean
StAR	Steroidogenic acute regulatory protein
TCF	T-cell factor
TGC	Trophoblast binucleate giant cell
TGF-β	Transforming growth factor, beta
TGF-βR	Transforming growth factor, beta receptor
TNC	Trinucleate cell
TP53	Tumour protein p53
β-TrCP	β-transducing repeat-containing protein

This is dedicated to my wife for her love and support in all my endeavours. Your unwavering support was essential in getting me to this point.

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INTRODUCTION

The development of placenta is a critical prerequisite for development of the embryo past the blastocyst stage. The placenta has a specialized pregnancy-specific structure that functions as a source of nutrients and hormones and protection for embryo/fetus throughout gestation. Placenta shape and form is largely variable among the mammals. The bovine placenta was described anatomically as synepitheliochorial (Wooding et al. 2008) that is initially formed by trophectodermal cells invasion to caruncular endometrium around 4th week of gestation to form villi or cotyledons. At the later stages of pregnancy, each caruncle and its associated cotyledon will be referred to as a placentome (Schlafer et al. 2000). Embryos that fail to accomplish proper placentation suffer from various morphologic defects and die. The complications found during normal gestation are rarely reported. However, since the introduction of assisted reproduction techniques (ART) such as in vitro production of embryos and nuclear transfer cloning, several reports have shown on low survival rate and high pregnancy failure due to abnormal placental development (Diskin et al. 1980; Hill et al. 2001; Heyman et al. 2002; Constant et al. 2006). A broad spectrum of anatomical and physiological anomalies was reported to be related to poor placentation in somatic cell nuclear transfer (SCNT) derived embryos.

It seems that the first trimester of gestation is a critical period for bovine SCNT embryos as the majority of implanted embryos are lost in this window (Willadsen et al. 1991) mostly accompanied with flattened cotyledons and abnormally shaped placentomes (Stice et al. 1996; Hill et al. 2000; Hashizume et al. 2002). Beyond day 90, placentome hypertrophy is the most frequent anomaly in bovine SCNT pregnancies (Hill et al. 1999;

DeSouza et al. 2001). Similar abnormalities were also reported in ovine SCNT gestations (DeSouza et al. 2001). Reduction in placental vascularisation was also reported in SCNT gestations (Palmieri et al. 2007). Although few bovine SCNT gestations reach full term, many give birth to overweight malformed neonates with placental abnormalities such as oedematous placenta, placentomegaly, hydroallantois and enlarged umbilical vessels (Hill et al. 1999; Wells et al. 1999; Constant et al. 2006).

In addition to abnormal gross placental morphology, changes in cellular and sub-cellular population were reported repeatedly in placenta of SCNT embryos (Hill et al. 2000; Hashizume et al. 2002; Arnold et al. 2006). Histologically, hypoplasia of trophoblastic cells, reduced numbers of the trophoblast binucleate cells (BNCs) and sub-epithelial haemorrhages were observed in different studies on SCNT pregnancies (Stice et al. 1996; Hashizume et al. 2002; Loi et al. 2006; Palmieri et al. 2007).

The atypical placentation in SCNT also endangers fetal well-being by alteration of normal placenta function. Many proteins involved in the regulation of feto-maternal interface such as bovine pregnancy-associated glycoproteins (bPAG) 1 and 9 and bovine prolactin-related protein (bPL) 1 are deregulated throughout pregnancy in SCNT placenta (Hashizume et al. 2002; Patel et al. 2004; Hirayama et al. 2008). Studies have demonstrated that the perturbations in steroidogenic activity of placenta in bovine SCNT pregnancies are related with some late gestational problems such as higher birth weight, abortion and prolonged gestations (Shah et al. 2007; Hirayama et al. 2008). Generally, there is aberrant molecular biology in SCNT pregnancies, as shown with an abnormal protein profile (Kim et al. 2005). Together, it seems that during high risk pregnancy, characterization of the

morphological and functional changes of fetal membranes is critical. Also, by defining the fetal growth parameters and morphologic characterization of the placenta, we will be able to predict through a standardized protocol the stages when the normal fetal development and wellbeing is compromised during clone gestations and to intervene medically in emergency cases to improve the overall efficiency of cloning in cattle.

Literature Review

ARTICLE I: Somatic Cell Nuclear Transfer Clones and Placental Anomalies in Cattle – a review

Status: In preparation

Somatic Cell Nuclear Transfer Clones and Placental Anomalies in Cattle – a review

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Abstract

In ruminants, somatic cell nuclear transfer (SCNT) can result in healthy offspring however, it is often associated with pathological changes in the fetus and the placental phenotype. The fetal membranes are essential in maintaining the fetus during pregnancy, any pathological changes of the placenta have potentially serious consequences for embryonic and fetal development and neonatal survival. The low efficiency of SCNT in producing embryos that develop into normal and healthy offspring has triggered research interest to understand mechanisms associated with morphological and functional placenta abnormalities to improve cloning technology. Although current evidence implicates aberrant reprogramming by epigenetic mechanisms of the donor chromatin as a cause, the association between morphological, functional and clinical abnormalities is poorly understood. Given the great variability in phenotype observed in SCNT, even with the same nuclear genetics, several aspects of cloning need to be explored. The present review summarizes current understanding of the normal placental structure in cattle and discusses developmental, morphological and functional abnormalities in placenta associated with SNT cloning.

Introduction

While still mobile in the uterus, the outermost layer of the embryo (the trophoblast) has acquires some specialization toward its eventual role in the transport of nutrients and fluids, synthesis of proteins and hormones, and excretion of waste products. The early embryo initiates communication with the mother to ensure maternal recognition of the pregnancy, achieve implantation, establish the interface for exchanges between maternal and fetal circulation, and alter the local immune environment. The feto-maternal interface, the placenta, is responsible for maintenance of the fetus, including sustaining the favorable nutrient partitioning essential to fetal growth and its physical protection. Any placental abnormality is expected to increase the risk of death of the embryo or fetus or compromise the life and development of the neonate.

The somatic cell nuclear transfer (SCNT) cloning opened a new chapter in placenta research field. This reproductive assisted technique has undeniable potential benefit for research of genetic and epigenetic mechanisms underlying developmental biology, aging, carcinogenesis (Meissner et al. 2006), generation of organs for xenotransplantation (Baguisi et al. 1999; Dai et al. 2002), developing transgenic animals for production of valuable recombinant proteins, saving genetically valuable animals (McClintock 1998) and for preserving endangered breeds and species (Loi et al. 2001). However, practical advantages are reduced by frequent developmental, morphological and functional abnormalities of the placenta. Clinical observations, macroscopic and histological evidence, gene expression studies and hormone profiles, all incriminate placental dysfunction as a substantial factor in

the failure of SCNT derived pregnancies. This gives fresh impetus to determine the association between placental dysfunction and pregnancy loss.

The purpose of this review is to summarize current knowledge on normal placental and discuss developmental, morphological and functional abnormalities in cattle associated with somatic cell nuclear transfer-derived gestation. Practical approaches to remediation rising from the knowledge of placental dysfunction should increase embryo and fetal survival and reduce economic losses.

Placental development

Embryogenesis in cattle has been well documented. At Day 7, the bovine embryo (Day 0 = fertilization) is in the blastocyst stage, characterized by a hollow central cavity and a plaque of cells lining in the inner blastocoel at one pole (inner cell mass; ICM), all surrounded by an outer layer of trophoblast cells (trophectoderm). The ICM develops into the embryo proper and the trophoblast cells into the fetal component of the placenta (McLaren 1972). At Day 8, a specialized cell layer (the endoderm) will grow from the ICM, inside the trophoblast cell layer and forms the yolk sac (Patten 1964). In addition, a third layer of cells (the mesoderm) grows from the ICM between the endoderm and the trophoblast layer. After hatching or emergence from the *zona pellucida* at 150 μm in diameter (Day 9), the growth of the embryo is rapid and reaches about 15 cm long, occupying the entire uterine lumen by Day 20 (Constant et al. 2006). This embryonic

elongation establishes the first direct contact between the embryo and the mother prior to implantation. By day 12, the trophoblast cell and mesoderm layers combine to form the chorion (Patten 1964). The chorion grows around the embryo forming a fluid-filled space around the embryo called the amniotic sac. Prior closure of the embryo abdominal wall, a new sac (allantois) extends between the yolk sac and the chorion. As the chorion extends out into the uterine lumen, the allantois grows rapidly and fuses with the chorion to form the chorioallantois (Schlafer et al. 2000). In ruminants, the outer layer of trophoblast cells (chorion) proliferates and differentiates into binucleate (BNC) and mono-nucleate cells (MNC) in a ratio of one to five (Wooding 1992). The MNCs synthesize placental interferon-tau (IFNt), a pregnancy recognition protein in cattle (Roberts et al. 1992) and BNCs synthesize several pregnancy associated proteins (Wooding et al. 1994).

Early during the process of implantation of the embryo (Day 20, apposition phase), the trophoblastic papillae (Guillomot et al. 1982) of the chorion (trophoblastic cells and connective tissues) enter into the uterine glands of the endometrium to achieve more intimate interaction and to anchor the primitive placenta to the uterus. This results in a diffuse and transitional placentation that enables the fetus to absorb the uterine secretion. This intimate contact between the placenta and the mother is first observed in the regions nearest the embryo and progressively continues to the tip of the elongated sac. By Day 25, the BNCs have migrated into the endometrium and fuse with uterine epithelial cells to form a multinucleated fetomaternal cell, a syncytium that is critical to villous formation and maintenance of a closer adhesion between microvilli and the trophoblastic cell membranes of the attachment phase (Wooding et al. 1980; Wooding 1992; Guillomot 1995). The

syncytial cells produce placental lactogen beginning on Day 18, pregnancy-associated glycoproteins (PAG's); beginning on Day 25. They also synthesize prolactin-related proteins (Wooding 1981; Kessler et al. 1991; Zoli et al. 1992; Green et al. 1998; Patel et al. 2004), and release them into the uterine connective tissue.

The number of BNCs within the trophoblast increases from Days 20 to 30 of the gestation (Morgan et al. 1989), whereas the interface between the outer layer of the chorion formed by the single layer of trophoblast cells and the endometrium is maintained as a simple epitheliochorial attachment. At Day 22 of gestation, the amniotic sac is formed by folding of the trophoblastic cells and mesodermal cells (inside) and begins to accumulate fluid, whereas the allantoic sac is completed at Day 24 and the attachment of the placenta by Day 27 (King et al. 1981; Schlafer, Fisher et al. 2000). The allantoic membrane is a thin and translucent and well vascularized by arteries originating from the aorta and umbilical veins. While the amniotic and allantoic membranes are formed, the third fetal membrane, the vitellin sac, disappears. Adhesion and attachment between the placenta and the endometrium becomes stronger by Day 30 (King, Atkinson et al. 1981; Guillomot 1995). The more intimate contact of the placenta with the maternal tissues induces a localized thickening on the fetal side that becomes the cotyledon (Li et al. 2005) where it covers preexisting uterine caruncles. The interdigitated chorionic villi and uterine crypts develop by Day 45 of gestation (King et al. 1979) and appear macroscopically as placentome; the union of the cotyledon and the caruncle. In cows, the placentation is classified as synepitheliochorial (Wooding 1992) because of the BNC-derived syncytium and the

epithelio-chorial interaction, representing large areas of simple apposition of maternal tissues known as the intercotyledonary space.

Morphology of the placenta

The surface of the endometrium in pregnant cow has four rows of specialized endometrial regions known as caruncles along the long axis of both horns, with a total of 60 to 80 convex and ovoid structures (Duello et al. 1986). Hydrostatic pressure of the allantoic and trophoblastic membrane against the uterine wall is believed to facilitate chorioallantoic attachment to the caruncle and stimulate the fetal chorion to vascularise and hypertrophy. As the cotyledons mature, the remodelling of the endometrium that leads to the development of the caruncles is necessary to accommodate the specialized folding of the chorioallantois. The surface of contact between maternal and fetal tissues is increased by the development of outgrowths on the surface of chorion, known as the villi. These chorionic villi consist of vascular mesenchymal cones surrounded by cuboidal trophoblastic and giant binucleate cells bring the fetal (allantoic) vessels into proximity with the maternal blood vessels. The temporal and spatial changes of the extra cellular matrix (ECM) during apposition, adhesion and attachment increase the complexity of placental formation and implantation process (Johnson et al. 2001; MacIntyre et al. 2002; Xiang et al. 2002).

Placentomes are larger and more numerous closer to the fetus and in the horn containing the fetus relative to those located in the uterine extremities and those found in the contralateral horn. Total placentome weight and length increase until Day 190, attaining

approximately 4.5 kg and 10 to 12 cm (Schlafer, Fisher et al. 2000) with most acquiring a mushroom-like shape with an occasional flat configuration (Miles et al. 2004). The total number of placentomes does not correlate with the increased fetal nutritional demands of late pregnancy suggesting that there is an alteration in the pattern of vasculature to increase feto/maternal exchange without an increase in placentome number (Leiser et al. 1997).

Placenta: an endocrine organ

The bovine placenta is an autocrine, paracrine and endocrine organ, in that it synthesizes a broad range of steroids and peptide hormones that regulate the development of the feto-placental unit (Gootwine 2004). Among hormones produced are steroids and protein factors including growth hormone (GH), insulin like growth factor-I (IGF-I), cytokines (Bauer et al. 1998) lactogenic hormones (PLs), and relaxin (Sjaastad et al. 2003). Placental protein hormones stimulate ovarian hormone production and fetal growth while contributing to mammary development and parturition. Growth hormone and IGF-1 have an anabolic effect and regulate fetal growth through cell growth and differentiation (Creasy et al. 2004). Relaxin, produced by both the placenta and ovaries, prevents uterine contractions during pregnancy and causes connective tissue in the cervix and pelvic ligaments to depolymerize and relax before parturition, facilitating expulsion of the fetus (Sjaastad, Hove et al. 2003).

Pregnancy in cattle depends, for the most part, on progesterone produced by the corpus luteum (Sjaastad, Hove et al. 2003) however; pregnancy can be maintained by the

placenta in the absence of ovarian progesterone after 200 days of gestation (Johnson et al. 1962; Estergreen et al. 1967; Johnson et al. 1981). Progesterone is synthesized within the placenta from circulating maternal cholesterol (Creasy and Resnik 2004), even though the total amount produced by the placenta seems to be small (Melampy et al. 1959). During steroidogenesis in the placenta, a series of catalytic reactions decrease the size of the basic 27-carbon-unit structure of the cholesterol to 21-carbon molecules (progesterone) (Thorburn et al. 1994). The bovine placenta has both P450scc and 3βHSD activity (Shemesh et al. 1989) in addition to StAR mRNA (Pescador et al. 1996; Verduzco et al. 2011). Progesterone is necessary for secretion of the histiotroph, comprised of various uterine proteins formation prior to completion of placentation, maintenance of myometrial quiescence, stimulation of mammary gland development and, suppression of immune rejection of the conceptus (Thorburn and Harding 1994). Other steroids, e.g. estrogens, are believed to be important in placental steroidogenesis. Some estrogens are mediators of implantation, uterine growth, mammary duct development, pelvic and cervical relaxation, induction of myometrial oxytocin receptors and, development of maternal behaviour (Thorburn and Harding 1994). The source of steroids in the ruminant placenta is not well known, although there is evidence that the mononucleate trophoblastic cells are the main source of placental estrone (Matamoros et al. 1994; Verduzco. et al. 2011).

The bovine placenta also produces a large family of glycoproteins named the pregnancy-associated glycoproteins (PAGs) which is specifically expressed by BNC of fetal cotyledonary tissue (Zoli, Guilbault et al. 1992). The PAGs are also known under a variety of names as pregnancy-specific protein 60 (Mialon et al. 1993) or pregnancy

specific protein B (Butler et al. 1982; Lynch et al. 1992). The PAGs are secreted continuously throughout gestation, with concentrations in maternal serum rising from Days 24 to 28 to the end of pregnancy and persisting after parturition (Sasser et al. 1986; Humblot et al. 1988; Humblot et al. 1988). From Day 33 of gestation, cows can be correctly diagnosed pregnant and non-pregnant by radioimmunoassay with about 87 % accuracy rate (Szenci et al. 1998). Therefore, a PAG assay can be used to detect and monitor pregnancy and to determine factors influencing late embryonic mortality (Humblot, Camous et al. 1988; Humblot, Jeanguyot et al. 1988). In the case of embryonic mortality, PAG concentrations were depressed before disappearance of the corpus luteum(Humblot, Camous et al. 1988; Humblot et al. 1991).

The synthesis and the secretory pattern of each PAG may differ as some proteins can be measured as early as Day 19 and others only appear by Day 45 of the gestation. Pregnancy-associated glycoprotein-1 can be measured between Day 20 and Day 30. Its concentration remains stable between Day 40 and 70 and slowly increases until Day 150, after which it increases rapidly to reach a peak about 20 days before calving. The plasma level remains elevated for about 3 months after calving because of its long half-life. Also synthesized by the BNCs, PL has a similar structure and function to the growth hormone and the relaxin (Constant, Guillomot et al. 2006). As expected for a protein derived from the fetus, the PL concentration of the mother is very low (1 to 2 ng/ml) compared to the fetal serum levels (Constant, Guillomot et al. 2006).

Placental consequences of SCNT cloning

Since the first bovine nuclear transfer (Prather et al. 1987) and the first demonstration that a nucleus derived from differentiated tissues from adult mammals may support fetal development to term (Wilmut et al. 1997), serious anomalies of gestation have been reported (Wells et al. 1999). In ruminants, these anomalies appear more extreme in clones produced from somatic cells relative to those produced from embryonic cells (Chavatte-Palmer et al. 2002). It is noteworthy that the rates of embryonic, fetal and perinatal survival are variable in cloned ruminant species (Baguisi, Behboodi et al. 1999; Lee et al. 2005; Galli et al. 2008). Although the specific pathologies vary among fetuses, the predominant cause of pregnancy failure associated with SCNT cloned embryos is believed to be associated with aberrant placenta, either morphologic or functional failures (Hill et al. 2000; Hill et al. 2001; Lee et al. 2004).

Four lines of evidence implicate abnormal placentas as a major cause of pregnancy failure and poor reproductive performance. First, the multitude, severity and the consistency of the placental lesions reported in multiple species could account for the high rate of pregnancy losses. Secondly, chimera studies with tetraploid blastomere NT cloned embryos in rodents further support the important role of placental anomalies in pregnancy losses (Eggan et al. 2001). Eggan (2001) showed that tetraploid blastomeres contributed only to formation of the placenta but not to the embryo proper in mice (Eggan, Akutsu et al. 2001). In this experiment, the neonatal SCNT mice cloned from embryonic stem cells had mean placental weight of 0.32 g that was significantly higher than those of tetraploid-produced pups (0.1 g) which were intermediate between SCNT and traditionally derived

pregnancies (Eggan, Akutsu et al. 2001). The third line of evidence can be found in similarities in placental and endometrial lesions between NT pregnancy and the interspecies pregnancies between the goat and the sheep (Hancock et al. 1968; Oppenheim et al. 2001). Early pregnancy losses were associated with reduced villi and crypts and reduced vascularity and presence of inflammatory cells in placentas (Hancock, McGovern et al. 1968; Allen 1982) which are also observed in NT pregnancies (Hill, Burghardt et al. 2000; Hashizume et al. 2002). The interspecies chimeric pregnancies seem to be similar to NTs in that in both gestations, the embryo is from a different genetic background and the maternal immunological rejection of the conceptus could result in implantation failure in early stage, or later, be manifest as placental anomalies (Dent et al. 1971; Oppenheim, Moyer et al. 2001; Davies et al. 2004). A fourth line of evidence is found in observations made on the effects of *in vitro* culture on placenta. The *in vitro* culture environment affects not only embryonic development and survival, but also alters placental development, morphology and function later in gestation (Farin et al. 2001). Placentas from *in vitro* produced (IVP) embryos that developed in the presence of serum were heavier, had a smaller caruncular surface, decreased villous volume density and reduced number of BNC compared to those produced *in vivo* (Farin et al. 2000). Alteration of placental development has been observed in SCNT cloned embryos with higher frequency and severity than in IVP embryos, providing further evidence for correlation of aberrant placentation with the low rates of gestational success in SCNT embryos (Cibelli et al. 1998; Hill et al. 1999; Hill, Edwards et al. 2001; Lee, Peterson et al. 2004).

Thus, the major impediment to the use of SCNT technique is its low efficiency, with less than 5% of SCNT pregnancies reaching term (Heyman et al. 2002). The majority of SCNT pregnancies in which the embryo survives and attaches to the uterus are lost between Days 30 and 90 of gestation (Willadsen et al. 1991) due to poorly developed placentomes (Stice et al. 1996; Hill, Burghardt et al. 2000; Hashizume, Ishiwata et al. 2002). Distinct placental morphology accompanies these losses. In the first trimester, cotyledons appeared flattened, abnormally shaped and reduced in numbers (Hashizume, Ishiwata et al. 2002). Placentomes in later pregnancy are frequently hypertrophied (Hill, Roussel et al. 1999; DeSouza et al. 2001). This hypertrophy is believed to be due, in part, to a mechanism to compensate for the reduced number of placentomes. This has been shown in an intrauterine growth restriction experiment in sheep where placental attachment sites were removed by carunclectomy, resulting in a significant increase in the size of the remaining placentomes (Robinson et al. 1979). In one bovine study, only 12 large functional cotyledons were found in the gravid horn of the uterus bearing a SCNT embryo (Hill, Edwards et al. 2001).

Alternatively, the mechanism of compensatory hypertrophy may be related to differential expression of transforming growth factors (TGF-βs) which are involved in all phases of development of the placenta that may alter the size of the placentomes in pregnancies derived from SCNT cloning. Between Days 50 and day 150 of gestation TGF-β1, TGF-β2, TGF-β3 mRNA abundance increased whereas expression of TGF-βR1 and TGF-βR2 receptors transcripts decreased significantly in SCNT placentomes, compared to pregnancies derived from artificial insemination (Ravelich et al. 2006). Consequently, the

placentomes of SCNT placentas may become resistant to the suppressive effects of the TGF-βs and become much larger.

The presence of smaller placentomes may be explained by a mechanism of compensation for the poor efficiency of larger placentomes by the formation of confluent smaller cotyledons around an already existing cotyledon (adventitial placentation). This has been observed in cases of chronic uterine conditions (Kennedy et al. 1993) and may be associated with development of hydrallantois, a condition observed frequently in SCNT cloned pregnancy. Enlarged umbilical vessels and edematous fetal membranes have also been reported in SCNT cloned fetuses (Willadsen, Janzen et al. 1991; Hill, Roussel et al. 1999; Wells, Misica et al. 1999).

In addition to the typical gross malformations of the placenta associated with SCNT pregnancies, the endometrial-trophoblast interface displays a reduced epithelial height with underdeveloped vascularity upon histological examination (Hill, Burghardt et al. 2000). Hashizume et al. (2002) reported the presence of fewer BNC at Day 60 in placenta of somatic NT pregnancy compared to controls derived by artificial insemination (Hashizume, Ishiwata et al. 2002). Similar observations were reported by Arnold et al. (2006) at Day 40 (Arnold et al. 2006). The question that arises is whether the reduced number of placentomes is a consequence of invasion of fewer BNC in cloned pregnancies, or whether fundamental differences in placentome formation result in few BNC. More investigation is clearly warranted. In addition to differences in placentome number, placental septae were more irregular and sparsely arranged in SCNT pregnancies (Hashizume, Ishiwata et al. 2002).

Morphometric analysis revealed that placentomes from IVP pregnancies have a smaller volume of fetal villi (Miles, Farin et al. 2004).

As proposed by Hill et al. (2000), the stage of pregnancy loss seems to be associated with different kinds of placental anomalies (Hill, Burghardt et al. 2000). Early pregnancy loss, occurring before complete placental establishment (less than 45 days) is believed to result from severely insufficient or delayed development of the chorionic epithelium and its vascularisation. The initial contact between the fetal trophoblast and the maternal caruncles does not induce the necessary hypertrophy of the fetal chorion and the formation of the normal cotyledon. Since fewer BNC migrate with the endometrial epithelium, the villous formation is perturbed and the maintenance and expansion of close adhesion between microvilli and the trophoblastic cell membrane is not maintained. This results in failure of the pregnancy. Fetuses dying between Day 45 to 90 of pregnancy appear to have compensated for earlier delays in chorionic development but do not develop a minimum threshold number of placentomes (Cross 2006) to allow a sufficient supply of nutrients, and gas exchange; therefore, the fetus eventually dies by starvation or other result of insufficient vascularization. This view is based on observations that, in the second trimester, fetal growth rate increases rapidly (Hubbert et al. 1972; Prior et al. 1979); the functional capacity of placentomes becomes critical because of the increased fetal demand for nutrients. If the compensatory mechanism (increasing placentome size) is not sufficiently rapid, the fetal demand is not met and the fetus died by the end of the second trimester (150 days of pregnancy). In addition to this, compensatory effects of increasing size, placentomes become resistant to the inhibitory effect of TGF-βs because of the

reduced number of receptors (TGF-βR1 and TGF-βR2), get even larger and allow pregnancy to be maintained. This escape strategy from growth inhibitory effect of TGF-β was reported in human cancers (Filmus et al. 1993; Markowitz et al. 1996). The loss of cell surface TGF-βR2 is correlated with functional resistance to growth inhibition by TGF-β in human colon neoplasia and result tumor progression (Markowitz et al. 1995). The compensatory growth of the reduced number of placentomes has some consequences that can result in pregnancy loss. These placentomes become so large in the last trimester that growth is disorganized and function of the tissue is impaired and eventually they undergo necrosis. This was observed in a case report of an SCNT cloned pregnancy, where fetal death occurred, the chorioallantois was mineralized and degenerating and necrotic cotyledons were observed (Hill, Edwards et al. 2001). In addition, the necrotic tissue, the vessels and the degenerating epithelium were mineralized.

Pregnancies with placental anomalies resulting from SCNT do not always result in fetal death. It is possible to predict calf size, and from there, their chance for survival so that the fetus can be delivered as soon as it is viable. If the calf grows an average of 0.35 kg/d in the last trimester (Prior and Laster 1979), birth of an extremely large fetus may be reduced and its chance of survival would be improved. The birth weight of the calf is predictable in the last trimester of pregnancy because of the linear correlation between minimal metacarpal or metatarsal thickness and body weight (Takahashi et al. 2001). These dimensions can be measured by transrectal ultrasonography, rendering it possible to detect oversized fetuses in cloned pregnancy and thus allowing intervention (Takahashi et al. 2005).

Conclusion

Placental membranes support the fetus throughout gestation however, anomalies in placental structure and function often result in SCNT pregnancy loss. These losses appear during the embryonic and fetal periods and are a major cause of decreased reproductive efficiency in cattle and a substantial cause of economic loss for the industry. Even though causes have been proposed to explain pregnancy loss, under field conditions, the etiology is often elusive. Currently, the frequent occurrence of placental anomalies in pregnancies obtained by SCNT derived pregnancy, points to an important role of placental dysfunction in pregnancy loss. Diagnostic tools like ultrasonography, hormonal profiles and molecular techniques could be used to monitor and study high-risk pregnancies derived from SCNT and better elucidate the role of placental dysfunction in pregnancy loss and potentially improve fetal survival.

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MINI-REVIEW: Role of Adherens junctions during placentation in ruminants

Introduction

In eutherian species, establishment of close contact with mother is necessary to maintain pregnancy. This feto-maternal interface has been defined as placenta and it develops throughout pregnancy in all mammals. This is a critical prerequisite for development of the embryo past the blastocyst stage. Embryos that fail to accomplish this will die because of various morphologic defects. The anatomical features of final form of this organ are the basics for placental classification. In ruminants, placentation is of the epitheliochorial and diffuse placentomal type.

The objective of this brief review is to highlight the new information on the role of adherens junction's proteins during placentation in eutherian mammals that is focused primarily on the ruminants.

Placental Development in Ruminants

The tissue source of placenta in eutherian mammals is the combination of two fetal membranes, the allantois and the chorion to form chorio-allantoic placenta. From the gross

morphology view, the placenta in ruminant is classified as syn-epitheliochorial placenta where the chorionic epithelium is in contact with the uterine epithelium. In epitheliochorial placenta structure, within the placentomes, the maternal epithelial layer is replaced by a syncytial layer that forms by migration and fusion of trophoblast binucleate giant cells (BNCs) to endometrial epithelium thus serving as a maternal-fetal barrier (Wooding 1992; Potgens et al. 2002).

In this type of placenta, the mononucleate trophoblast cells (MNCs) differentiate to trophoblast binucleate giant cells (BNCs, also referred as TGCs). This procedure is accompanied by an exit from the mitotic cell cycle and onset of endoreduplication. The BNCs then rapidly lose contact with the basement membrane. After maturation takes place, the tight and gap junctions disappear leaving only a desmosomal-like contacts with the mononucleate cells of the epithelium (Lawn et al. 1969; Boshier et al. 1977). A BNC can fuse with the apex of a single uterine epithelial cell to form a hybrid trinucleate cell (TNC) and further BNCs can fuse apically to extend this minisyncytium to five or seven nuclei or beyond (Wooding et al. 2008). With respect to polyploidy and migratory/invasive properties, BNCs seem to be analogous to extravillous cytotrophoblasts in humans and trophoblast giant cells in rodents (Hoffman et al. 1993). A similar ratio of BNCs to MNCs in the trophectoderm tissue was determined in all ruminant placentomes (15–20% BNCs) (Wooding et al. 2008). BNCs are vital in maintenance of pregnancy, as they produce hormones, including the placental lactogen/prolactin-related protein family and pregnancy-associated glycoprotein family (Wooding et al. 1994). The mechanism of BNC formation is poorly understood, however, this process accompanies by remodelling in cytokeratin

filaments (Wooding et al. 1994), tight junctions (Morgan et al. 1983), integrin subtypes (Pfarrer et al. 2003) and altered expression of adherens proteins (Nakano et al. 2005).

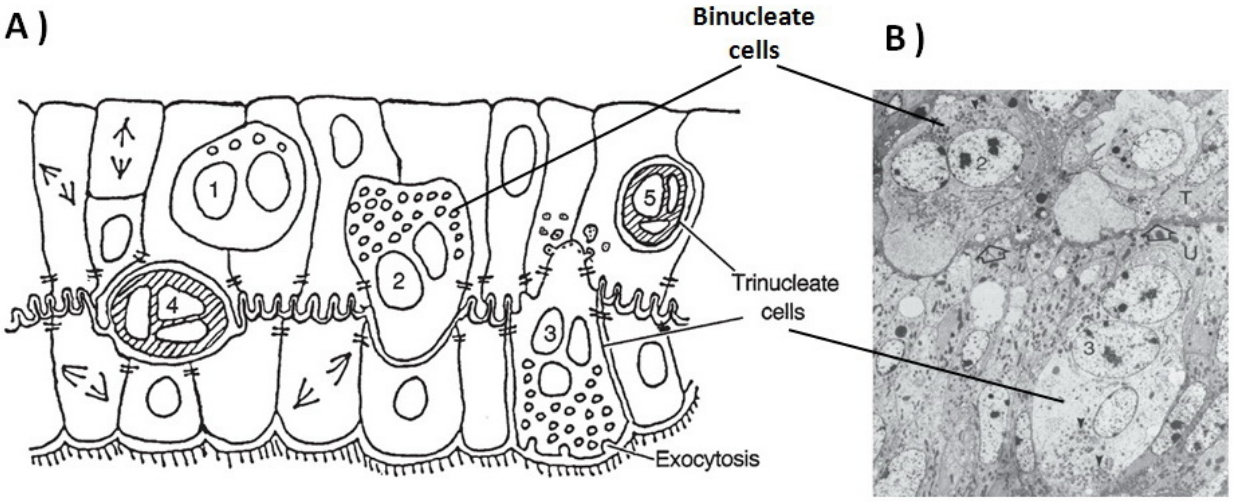

Figure 1 - Illustration of bovine placenta.

A schematic model (A) and micrograph (B) of bovine placenta (20 dpc). The granulated binucleate cells (BNCs) (1 and 2) migrate from trophectoderm (T) to fuse to caruncular epithelium (U) and form trinucleate cells (3). Micrograph magnification: 2800 x. Ref.: (Wooding et al. 2008)

Adherens junction's structure

Epithelial cell–cell junctions are formed by apical adherens junctions, which rely on the cadherin-catenins complex that interacts with the actin cytoskeleton. To maintain tissue integrity during its remodelling throughout development, cell–cell junction stability and dynamics must be crucially regulated. The molecular components of the adherens junction (cadherin-catenins) not only mediate the basic adhesiveness of cells but also they participate in other cellular events such as intercellular signalling (Gottardi et al. 2001; Perrais et al. 2007), cell sorting (Wei et al. 2005) and polarity (Nejsum et al. 2007; Wang et al. 2007).

Cadherins

The cadherins constitute a superfamily of calcium-dependent cell adhesion glycoproteins that interact on opposing cell surfaces which result in weak cell–cell adhesion. They play a major role in development and tissue morphogenesis (Larue et al. 1994; Takeichi 1995), regulating apoptosis (Makrigiannakis et al. 1999), cell differentiation and tissue polarity(Ong et al. 1998). The well-known members of this superfamily are classical cadherins that are found in most solid tissues of the body (Tepass et al. 2000). Classical cadherins are named according to the tissues from which they were first isolated such as E-, VE -, and R-cadherins were derived from epithelial, vascular endothelial and retinal tissues, respectively. The structure of classical cadherins is defined as single-pass trans-membrane protein that interacts with different cytoplasmic proteins. The formation of trans-oligomers from the multiple cis-dimers (Brieher et al. 1996) between cadherins on

opposing cell surfaces is mediated by a calcium-dependent protein conformation (Pertz et al. 1999) and create a weak binding between neighbour cells(Chen et al. 2005). The cell–cell adhesion would become stronger by lateral clustering of cadherins which depends on the linkage of E-cadherin–β-catenin complex to actin-binding protein α-catenin (Aberle et al. 1994; Huber et al. 2001).

Among classical cadherins, E-cadherin is the most commonly studied in the context of stable adhesions that forms the adherens junctions in epithelial cells. E-cadherin stabilizes the cell architecture and its altered expression is associated with the induction of epithelial–mesenchymal transition that is observed in tumour-cell invasion and metastasis (Christofori et al. 1999). Transfection of non-epithelial cell lines with E-cadherin induced polarization and an epithelial phenotype, thus confirming a vital role for E-cadherin in epithelial differentiation (Nagafuchi et al. 1987; Marrs et al. 1993). The cytoplasmic region of E-cadherin is the most highly conserved portion of this protein and it is the region that interacts with three major cytoplasmic signaling proteins α-catenin, β-catenin, and γ-catenin (Ozawa et al. 1989). The carboxy-terminal of cytoplasmic structure binds to β-catenin or γ-catenin which in turn binds to α-catenin (Ozawa et al. 1990; Ozawa et al. 1992; Shapiro et al. 2009). Among the other catenins, E-cadherin is associated to β-catenin from when they synthesize in endoplasmic reticulum and the two proteins move together to the cell surface (Hinck et al. 1994). It seems that β-catenin prevents proteosomal destruction of cadherin, as any disruption of this interaction results the proteosomal degradation of E-cadherin (Chen et al. 1999).

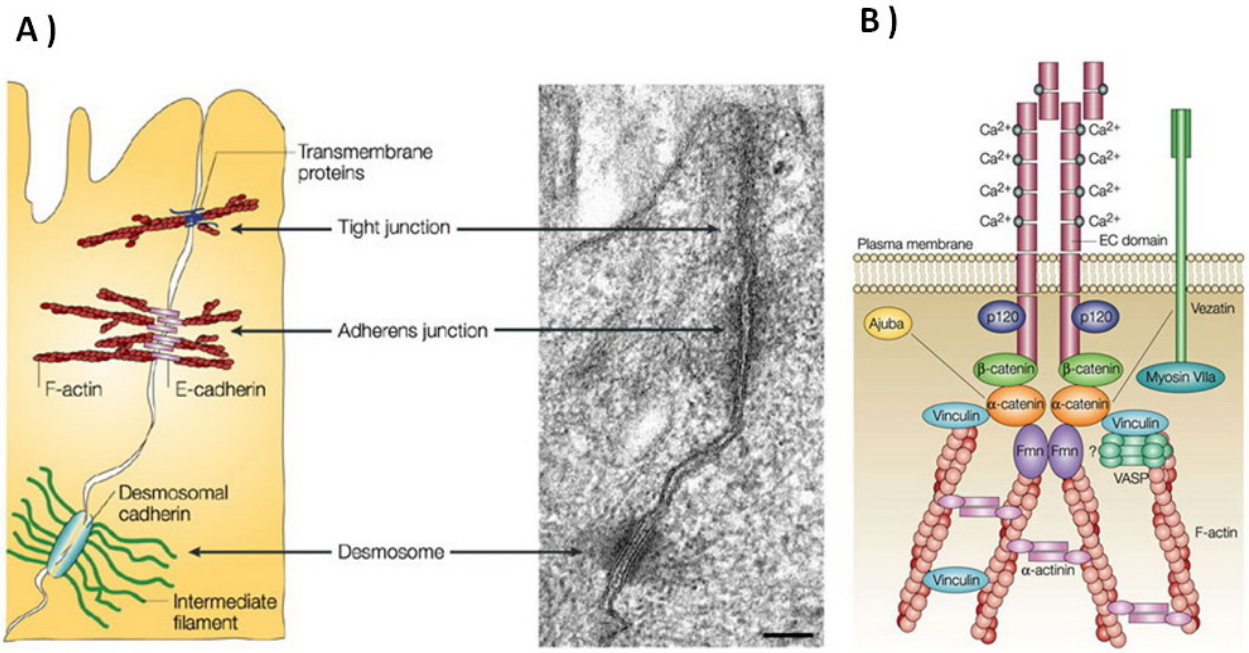

Figure 2 - Adherens junction's structure

A) Adherens junctions is one type of intercellular junction in epithelial cells. They form a homophilic interactions between E-cadherin molecules which are connected to actin cytoskeleton through catenins complex. B) A schematic presentation of adherens junction. The E-cadherin molecule consists on Ca^{2+} dependent extracellular (EC) domains that interact with E-cadherin molecules from neighbour cells. The cytoplasmic domain of E-cadherin binds to α-catenin through β-catenin which is central in recruiting a number of cytoskeletal proteins. Ref.: (Kobielak et al. 2004)

Catenins

Classical cadherins have a conserved cytoplasmic domain with the capability to form complexes with the catenins that are α-, β- and γ- catenins (Plakoglobin) and p120 (Aberle et al. 1996). The evidences indicate that β-catenin interacts more directly with the cytoplasmic domain of E-cadherin than does α-catenin (Ozawa et al. 1992) whereas α-catenin mediates the interaction with actin filaments (Ozawa et al. 1990).

Plakoglobin (γ- catenin), another member of this family, is the only component that participate in the structure of both desmosomes and adherens junctions (Cowin 1994; Mathur et al. 1994). At adherens junctions, plakoglobin makes a link between classical cadherins and α-catenin, which in turn connects the complex to the actin cytoskeleton both directly and through α-actinin (Jou et al. 1995; Rimm et al. 1995). Another catenin, p120, which was originally identified as a substrate of Src family tyrosine kinases (Reynolds et al. 1992) that may participates in regulating the adhesive function of cadherins.

Among the catenins, β-catenin is widely studied as it is not only constitutes the adherens complex but also regulates vital cell events such as proliferation, migration and differentiation by acting as a transcriptional regulator for several genes in the nucleus (Goldstein et al. 2006; Li et al. 2006). This protein is highly conserved among species. The primary structure of the 781 amino acid β-catenin consists of an amino-terminal region of about 150 amino acids, a central ~520 residue domain composed of 12 armadillo (arm) repeats, and a carboxy-terminal 100 residue region. E-cadherin binds to the arm domain by casein kinase II (CKII) and GSK3-β modulated phosphorylation (Lickert et al. 2000),

whereas α-catenin binds to residues 118-149, just before the start of the arm domain (Pokutta et al. 2007; Shapiro et al. 2009).

The cytosolic pool of β-catenin is continually marked for destruction by a cascading phosphorylation mechanism on the NH2 terminus. CK1α phosphorylates β-catenin at serine 45, and this priming phosphorylation results in subsequent phosphorylation by Glycogen synthase kinase 3β (GSK3-β) at residues 41, 37, and 33 (Yost et al. 1996; Liu et al. 2002). The phosphorylated β-catenin at residues 37 and 33 is then ubiquitinylated by the β-TrCP E3-ligase complex and subsequently goes under proteolytic destruction by 26S proteasome (Hart et al. 1999). WNT signalling activation disrupts this process by enhancing the phosphorylation of GSK3-β that is leading to accumulation of free and unphosphorylated β-catenin in the cytoplasm which then translocates to the nucleus. Nuclear β-catenin links the lymphoid enhancer factor 1 (LEF1)/ T-cell factors TCFs proteins to specific chromatin remodeling complexes that promotes changes in the transcriptional machinery leading to activation of several target genes(Willert et al. 2006). Several genes have been identified as the target of β-catenin- LEF1/TCF transcriptional regulation such as cyclin D1 (Takahashi-Yanaga et al. 2008) and c-Myc (Wilkins et al. 2008) which have crucial roles in cell growth, proliferation and differentiation during embryogenesis (Varlakhanova et al. ; Wang et al. 2006; Bryja et al. 2008), WNT/β-catenin pathway also control the cell remodelling, adhesion and migration through regulation of proteins such as CD44 (Wielenga et al. 1999), fibronectin (Gradl et al. 1999) and laminin-5γ2 (Hlubek et al. 2004).

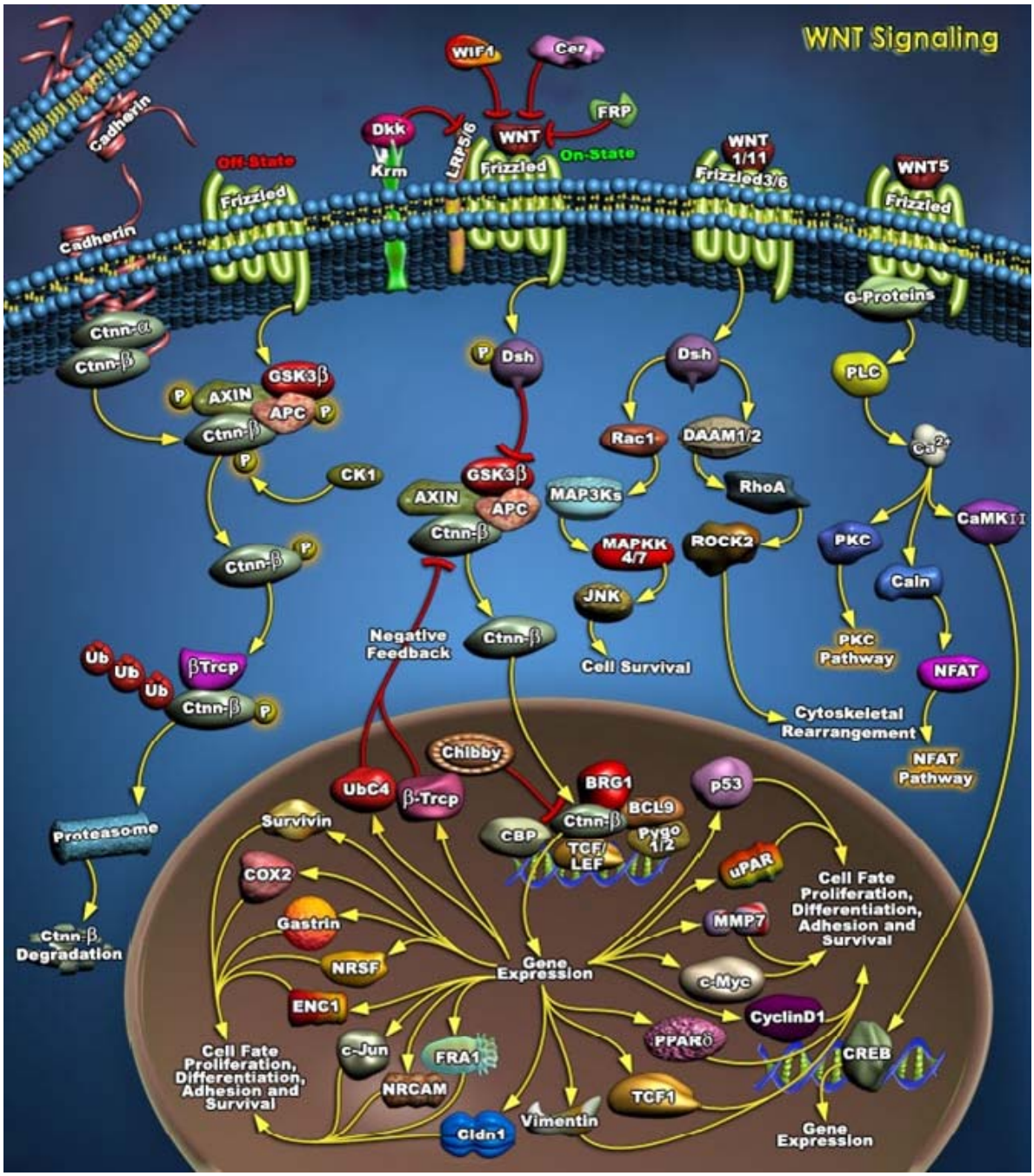
WNT Signaling
WIF1
Cer
FRP
Dkk
Krm
LRP5/6
WNT
Frizzled
On-State
Off-State
Frizzled
Cadherin
Cadherin
WNT
1/11
Frizzled3/6
WNT5
Frizzled
G-Proteins
Ctnn-α
Ctnn-β
GSK3β
P
AXIN
APC
P
Ctnn-β
P
CK1
P
Dsh
Dsh
Rac1
DAAM1/2
PLC
Ca2+
GSK3β
AXIN
APC
Ctnn-β
MAP3Ks
RhoA
PKC
CaMKII
Caln
MAPKK
4/7
ROCK2
JNK
Cell Survival
PKC
Pathway
NFAT
Ctnn-β
P
Ub
Ub
Ub
βTrcp
Ctnn-β
P
Negative
Feedback
Ctnn-β
Cytoskeletal
Rearrangement
NFAT
Pathway
Chibby
UbC4
β-Trcp
BRG1
p53
Survivin
Ctnn-β
BCL9
CBP
Pygo
1/2
TCF/
LEF
Proteasome
COX2
uPAR
Cell Fate
Proliferation,
Differentiation,
Adhesion and
Survival
Gastrin
MMP7
Ctnn-β
Degradation
NRSF
Gene
Expression
c-Myc
ENC1
CyclinD1
c-Jun
FRA1
PPARδ
CREB
Cell Fate
Proliferation,
Differentiation,
Adhesion and
Survival
NRCAM
TCF1
Vimentin
Cldn1
Gene
Expression

Figure 3 - Canonical WNT signalling pathway.

In the absence of WNT proteins free β-catenin molecule is phosphorylated by a complex (AXIN, APC and GSK-3β) and then is ubiquitinated by the β-Trcp and subsequently degraded by the proteasomes. At the "OnState" status, WNT ligands associate with appropriate receptors (Frizzled and LRP-5/6 co-receptors) and recruit AXIN to the plasma membrane. This will release β-catenin in non-phosphorylated form that subsequently translocates into the nucleus to promotes transcription of WNT target genes upon binding to TCF/LEF. Ref. : *Ambion Applied Biosystem – GeneAssist Pathway Atlas*

Role of adherens junction's proteins during placentation

It has been shown that during embryogenesis in mouse E-cadherin is essential at early stages when E-cadherin knockdown mice embryos failed to form trophectoderm epithelium or blastocyst cavity at the blastocyst stage (Larue et al. 1994). At gastrulation E-cadherin expression is suppressed in mesodermal cells with no significant alteration in expression of catenins (Butz et al. 1995).

It was reported that genomic replacement of E-cadherin (*CDH1*) by N-cadherin (*CDH2*) cDNA in mouse embryos results the failure in formation of intact trophectoderm (Kan et al. 2007) but if a tagged *CDH1* cDNA is knocked into the Cdh1 (E-cadherin) locus, the embryos form proper trophectoderm, implant and undergo both gastrulation and neurulation but they die later on because of incapability to establish a proper connection to maternal blood accompanied by increased apoptosis and reduction in cell numbers in placental tissue (Stemmler et al. 2010). Immune-localization of E-cadherin and beta-catenin in pre-attachment bovine embryos revealed that in trophectoderm cells their expression is restricted to the basolateral membrane while they maintain apolar distribution in the inner cell mass (Barcroft et al. 1998).

An important role of β-catenin during embryonic development was confirmed in a range of studies. In one experiment, β-catenin null-mutant mouse embryos formed blastocysts but with reduction in cell adhesion that resulted in detachment of ectodermal cells (Haegel et al. 1995). Also, similar study revealed defects in anterior-posterior axis formation at embryonic day 5.5 (Huelsken et al. 2000). The epithelial-mesenchymal

transition. an essential event during placentation in mammals with the invasive type of trophoblast (Vicovac et al. 1996), is under control of WNT signalling and cadherin-mediated adhesion and β-catenin as an essential molecule in both of them play a crucial role in its regulation (Heuberger et al. 2010). However, cytoplasmic and nuclear expression of β-catenin in mature bovine binucleate cells compared to peripheral expression immature BNCs and MNCs revealed that WNT signalling could play an important role during maturation of BNCs, especially when we considered that some of WNT target genes such as Cyclin D1 and c-Myc that control the cell cycle, might involve in endoreduplication process in BNCs (He et al. 1998; Tetsu et al. 1999; Nakano et al. 2005).

Conclusion

This review outlines the role of adherens proteins, more specifically E-cadherin and β-catenin during placentation. Regulation of these proteins and the signalling events that follow downstream, play a profound role in the control of differentiation and survival of trophoblast binucleate giant cells (BNCs), normal development of the placenta to establish normal and healthy maternal-fetal contact. A better knowledge from trophoblast proliferation, migration and attachment events and their molecular regulation could help us in understanding of pathological anomalies in pregnancies in which trophoblast function is impaired.

HYPOTHESIS/OBJECTIVES

Problem

High fetal and prenatal morbidity and mortality encountered in cloned animals could in part be explained by placental deficiency (Hill et al. 2000; DeSouza et al. 2001). The placental insufficiency could reach a point where placentomes are unable to meet the nutritional requirements of the developing fetus. Different morphological anomalies in placenta were reported to be related to somatic cell nuclear transfer (SCNT) such as poor vascularisation (Hill et al. 1999; Hill et al. 2000) and placental formation and low placentome numbers (Hill et al. 2001; Lee et al. 2004). The bovine placenta is also an autocrine, paracrine and endocrine organ that synthesizes a broad range of steroids and peptide hormones (Battaglia et al. 1986) and any morphological alteration could affect the placental function and indirectly compromised the fetus survival rate during the pregnancy and in the prenatal period (Shah et al. 2007). So, patho-morphological and -physiological characterization of SCNT placenta not only provide experimentally valuable attributes that can be exploited to better understand the biology of the placenta but also can help us to develop new preventive treatment for SCNT-derived placental defects and to enable a better gestational outcome for cloned animals.

Hypothesis

The poor efficiency in cloning animals by nuclear transfer is associated with morphological and functional abnormalities of the placenta that eventually lead to fetal distress and mortality or postnatal morbidity.

Objectives

1- To characterize morphology of fetal membranes changes during gestation in SCNT cloned animal in order to identify placental anomalies for early diagnosis and early medical interventions to improve the overall efficiency of cloning in cattle and to establish the most critical period in SCNT pregnancies when the viability of the fetus and/or the dam is threatened.

2- To assess the pattern of changes in concentration of four placental related hormones (P4, E1S, E2 and PSPB) in maternal circulation during the last two trimesters of pregnancy and to investigate if these hormones could be used as an early indicator of problematic gestation in later stages in recipients cows bearing SCNT fetuses.

3- To evaluate the expression of two constructive epithelial cell adhesion molecules (E-cadherin and β-catenin) during early placentation window (Day 40) and determine how these factors affect the essential cell signalling and cell-cell adhesions involved in trophoblast proliferation and differentiation.

MATERIALS, METHODS AND RESULTS

ARTICLE II: Ultrasonographic and histological characterization of the placenta of somatic nuclear transfer-derived pregnancies in dairy cattle

Status: Published

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Ultrasonographic and histological characterization of the placenta of somatic nuclear transfer derived pregnancies in dairy cattle

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Abstract

The high incidence of pregnancy loss and prenatal morbidity and mortality in cloned animals may be due to placental insufficiency, thereby compromising fetal survival. Our objective was to characterize morphological changes in fetal membranes of cloned bovine pregnancies. Two groups of cows with cloned fetuses, produced by two cloning techniques, a commercial group (N=16) and a hand-made group (N=4), and control fetuses derived from traditional embryo transfer (N=6) or AI (N=6), were compared at various stages of gestation (Days 80, 120, 150, 180, 210, and 240; Day 0 = estrus). Thickness and shape of the amniotic membrane, placentome shape and length, umbilical cord shape and diameter, and fetal fluid echodensities were assessed by ultrasonography, and the placenta was evaluated histologically. Only eight (40%) of cloned pregnancies reached term and seven calves (35%) were alive at birth. Both placentome length and umbilical cord diameter were larger ($P<0.05$) in clones than in normal fetuses at all stages of gestation. Amniotic membrane abnormalities (Day 120) including focal edema and the presence of a series of nodules were detected in 38% of the clones and were always accompanied by hyper-echodense spikes or irregularities (detected ultrasonographically) around the umbilical cord. Histopathology revealed degenerate inflammatory cells, edematous chorioallantoic membranes, and decreased epithelial thickness. We inferred that these morphological anomalies of placentomes compromised fetal development, and we conclude that ultrasonographic monitoring of pregnancies enabled characterization of changes in the placentae and may be useful to assess fetal wellbeing.

Introduction

The placenta maintains the fetus by sustaining favorable nutrient partitioning and critical endocrine functions. Fetal survival and development during pregnancy depends on appropriate morphological and functional development of the placenta (Wooding 1992) and placental abnormalities are frequently associated with abortion or perinatal death. Assisted reproductive technologies (ART) in cattle have been associated with aberrant placental phenotypes and high rates of pregnancy loss (Wells et al. 1999). Nuclear transfer (NT) cloning in particular is associated with poor reproductive efficiency and is characterized by low pregnancy rates, high pregnancy loss and a plethora of fetal and placental abnormalities (Wells, Misica et al. 1999; Sakai et al. 2005). The overall efficiency of NT is typically < 5% (Hashizume et al. 2002). Heyman et al. (2002) reported fetal losses of 33 to 43% with transfer of cloned bovine embryos (Heyman et al. 2002). Anomalies appeared more extreme in clones produced from somatic cells relative to those produced from embryonic cells (Chavatte-Palmer et al. 2002; Farin et al. 2006). Although the specific fetal pathology varies, the predominant cause of pregnancy failure associated with NT cloned embryos in cattle is believed to be atypical placenta with an attendant alteration in morphology and gene expression, leading to metabolic anomalies (Hill et al. 1999; Hill et al. 2000; Hill et al. 2001; Ravelich et al. 2004; Farin, Piedrahita et al. 2006).

The majority of established NT pregnancies in cattle are lost between Days 30 and 90 of gestation, in association with poorly developed placentomes (Stice et al. 1996; Hill, Burghardt et al. 2000; Hashizume, Ishiwata et al. 2002). In the first trimester, NT derived placentomes have abnormal shapes and are present in reduced numbers (Hashizume,

Ishiwata et al. 2002). When pregnancy is maintained, placentomes were frequently hypertrophied (Hill, Roussel et al. 1999; DeSouza et al. 2001). Enlarged umbilical vessels and edematous placentae have also been reported (Willadsen et al. 1991; Hill, Roussel et al. 1999; Wells, Misica et al. 1999). In addition to the typical gross placental malformations, the endometrial-trophoblast interface displays a reduced epithelial height and underdeveloped vascularity (Hill, Burghardt et al. 2000).

As most literature has focused on dysfunction at the time of implantation in NT surrogate dams, the present longitudinal study was undertaken to assess placental abnormalities that could be potentially harmful for maternal and fetal well-being. The objectives were to: 1) establish baseline ultrasonographic information for evaluation of placentae in cloned pregnancies and characterize (with ultrasonography and histology) morphological changes throughout gestation; and 2) establish the most critical period in NT pregnancies when the viability of the fetus and/or the dam is threatened.

Materials and methods

Animals

All procedures were performed in accordance with the Canadian Council of Animal Care recommendations and approved by the University of Montreal Committee on Animal Care and Experimentation. Embryo recipients were Holstein heifers, 15 to 18 months old, average body condition score of 3.5 (scale, 1 to 5 (Heinrichs 1985)), and free of bovine viral diarrhea, neosporosis, and leukosis. All were raised in a free stall barn and fed

concentrate (2 kg/head/d), with *ad libitum* access to hay. Frequent observations confirmed the occurrence of regular estrous cycles in all recipients. The heifers were treated with 500 μg of cloprostenol (Estrumate, Schering-Plough, Pointe-Claire, QC, Canada) I.M. to synchronize estrus for embryo transfer or AI. Following detection of estrus, heifers were inseminated, received a frozen-thawed or a fresh cloned embryo. On Day 7 of the estrous cycle (estrus = Day 0), embryos were transferred non-surgically into the uterine horn ipsilateral to the CL. The AI control group (N=6) was composed of Holstein heifers that were inseminated with semen from Holstein bulls.

Embryo production

All embryos transferred to control group recipients were produced by *in vivo* fertilization, frozen after collection, and thawed before transfer using the one-step technique (Voelkel et al. 1992).

Two groups of clone embryos were included in this study. The first group was produced by a proprietary commercial cloning technique. Briefly, cumulus oocyte complexes (COCs) were aspirated and matured in TCM-199 with 10% fetal bovine serum (FBS) for 20 h in estradiol and FSH. Cumulus was removed via a small-bore pipette and rinsed several times in a proprietary medium (C4). Enucleation was carried out using a fine tipped glass pipette from a 15 cm capillary tube, proprietary medium (B0), Cytochalasin B, and UV fluorescence. Donor cells from one of the three cell-lines (bulls A, B and C) was transferred in B0 medium, with the transferred cell physically placed between the zona and oolemma by a fine-drawn pipette. Oocyte/cell complexes were treated with ionomycin and

immediately electrofused by a 2.4kV/cm pulse. Activation via cytochalasin B and cycloheximide for 6 h was followed by incubation for 7 d in C4 and C5 media. Resultant embryos were either transferred to stimulated recipient animals or cryopreserved for other studies.

The second group was produced by our laboratory using a hand-made cloning technique (Vajta et al. 2006) with some modifications. In brief, the cumulus oocyte complexes (COCs; diameter, 2 to 7 mm) were aspirated from abattoir-derived bovine ovaries. During in vitro maturation, COCs were incubated in TCM-199 (Gibco BRL) supplemented with 10% FBS, 50 μg/ml LH (Ayerst, London, ON, Canada), 0.5 μg ml^{-1} FSH (Folltropin-V; Vetrepharm, St-Laurent, PQ, Canada), 1 μg/ml estradiol 17-β (Sigma-Aldrich, St. Louis, MO), 22 μg/ml pyruvate (Sigma-Aldrich), and 50 μg/ml gentamicin (Sigma-Aldrich) for about 20 hours. Then cumulus cells were removed by 2 min shaking (vortex) of the COCs in PBS and 0.2% hyaluronidase (Sigma-Aldrich). After removal of zona pellucida, oocytes were washed in TCM supplemented with FBS 20% for 3 minutes then treated in 0.4 μg/ml demecolcine for at least 30 minutes followed by culturing in medium supplemented with 5 μg/ml cytochalasin and FBS 10%. The oocytes manually bisected in a stereomicroscope with help of a micro blade. Adult fibroblasts obtained by skin biopsy from one bull (designated bull D) used as nuclear donor cells. They were thawed at 37 ˚C, immediately washed in 10 ml of culture media (DMEM, supplemented with 10% FBS and 0.5% antibiotics), and then placed in a 50 μl of the culture using the same media. Nuclear transfer was performed using confluent cells that were maintained in culture for 3–5 passages. Cytoplasts were then placed individually in a 50 μl drop

containing 500µg/ml of phytohemagglutinin (Sigma-Aldrich) for about 3 sec and then quickly positioned over a single donor cell placed at bottom of the dish. After attachment of the donor cell, the cytoplast-somatic cell pairs were placed in 0.3 M mannitol solution containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$ and exposed to a 1.2-kV electric pulse lasting 70 millisec. After electrical stimulation, couplets were washed and cultured individually in 10µl drops of 6 DMAP (Sigma-Aldrich) for 3 h.

After 6 DMAP treatment, reconstructed oocytes were washed again and then cultured in 40µl drops of SOF modified medium supplemented with 0.8% BSA-V fatty acid free (Sigma-Aldrich) under equilibrated mineral oil at 39 ˚C in a humidified atmosphere of 5% CO_2 and 5% O_2. Reconstructed embryos were cultured for a period of 8 days in culture.

Ultrasonographic monitoring

All recipients were examined for the presence of a viable fetus on one occasion between Days 30 and 40, using transrectal ultrasonography (ALOKA 900, Alliance Medical Inc., Montreal, QC, Canada) equipped with a 5.0 and 7.5 MHz transducer. Pregnancy was re-confirmed by ultrasonography at Day 60 and experimental monitoring began at Day 80. At Day 120, 150, 180, 210, and 240 recipients further underwent transabdominal ultrasonography using a 3.5 MHz probe (Alliance Medical Inc.). The viability of the fetus and ultrasonographic aspects of the placenta were monitored and the examinations were recorded with a digital video camera (Sony, DCR-HC90 Handycam, Tokyo, Japan) for subsequent analysis.

To optimize image quality, the recipient's right ventral abdomen, from the xyphoid to mammary glands was clipped and washed with alcohol before carboxymethylcellulose gel was applied. Fetal heartbeat and movement were observed to establish fetal viability; placentome size, umbilical cord diameter, thickness of the chorioallantoic and amniotic membranes was estimated in three different areas: transrectally close to the anterior cervical os and at the bifurcation of the pregnant horn and by the transabdominal examination of the distal portion of the pregnant horn. The length (maximum) of 12 placentomes and two independent measurements of amniotic thickness and umbilical diameter were measured. Qualitative aspects of the amniotic and allantoic fluids were recorded and graded on a scale of 0 to 4, with 0 indicating homogeneous and hypo-echodense fluid to 4 indicating a heterogenic and hyper-echodense fluid (with particles). In addition, placentome shape and appearance, presence or absence of edema and any other abnormalities in membranes and umbilical cord were recorded. Hydroallantois was diagnosed by abdominal distention on physical examination and by excessive accumulation of allantoic fluid and difficulties in visualizing the fetus in the uterine cavity by ultrasonography. When fetal or placental abnormalities were associated with severe deterioration of the general condition of the recipient, the dam was euthanized, delivered by cesarean section or aborted. Uterus, placenta and fetal samples were recovered for further examination.

Calving

Cloned calves (Days 275 to 278) were delivered by caesarean section 24 to 36 h after the parturition was induced with 25 mg of dexamethasone 21 phosphate

(Dexamethasone 5, Vetoquinol, Joliette, QC, Canada) and 25 mg of dinoprost (Lutalyse, Pharmacia, Orangeville, ON) given I.M. The same protocol was used to induce abortion.

Tissue collection

Samples from placentomes, amniotic and allantoic membranes were collected from aborted recipients (NT group), at the abattoir (NT and control groups), at parturition (control group) and during caesarean section (NT group), at various times during gestation (Days 80, 120, 150, 180, 210, and 240). A 5 mm-thick slices were obtained from different regions of the placentomes, from the bottom of crypts (maternal side) to the surface (fetal side). Samples were fixed in 10% formalin, dehydrated and embedded in paraffin wax. Sections of 4 μm were cut and stained with haematoxylin-eosin-saffran (HES) for histological analysis.

Experimental design and statistical analysis

Four groups of pregnant recipients (G1=AI embryos, n=6; G2=frozen embryos, n=6; G3=commercial clones, n=16; and G4=Hand-made clones, n=4; Table 1) were monitored at six stages of gestation (Days 80, 120, 150, 180, 210, and 240). For quantitative parameters, a mixed linear model with age of gestation (as the within-subject factor) and group (as the between-subject factor) as main effects and age X group interaction, followed by Tukey's post-hoc test, was used as the statistical model. Intra-uterine location of placentomes was included in the model. Repeated-measures logistic

regression, accompanied by Chi-square and Cochran-Mantel-Haenszel tests, were also performed for qualitative parameters for each stage of pregnancy. Differences between groups and associations were considered significant if $P<0.05$. All analyses were performed with SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Based on preliminary statistical analyses, none of the factors, with the exception of amniotic membrane thickness differed significantly among controls (pregnancies derived by AI and embryo transfer) and the two cloned groups. Therefore, data from each pair for those parameters were merged to create one control group (AI + frozen embryo transfers, N=12) and one cloned group (commercial and hand-made clones, N=20). For amniotic membrane thickness, all four groups were considered separately in the statistical model.

All heifers but one from the control group had an apparently normal gestation and normal delivery. From the total number of cloned embryo transfers (N=47), the pregnancy rate was 49% (N=23) by Day 30 and 43% (N=20) by Day 60. Most of the losses confirmed at Day 60 were predicted at Day 30, due to increased echodensity of uterine fluid, the abnormal appearance of the embryo proper and the presence of free fetal membranes floating in the uterine fluid. Of 20 NT pregnancies confirmed at Day 80, only eight (40%) reached Day 240 (Table I, Fig 8). The 12 pregnancies that did not proceed to term were terminated for humane reasons by euthanization (at day 150 (N=1), 210 (N=1) and 240 (N=2)), cesarean (at day 210 (N=1), and 240 (N=2)) or abortion (at day 150 (N=3), 180

(N=1) and 210 (N=1)). Among the NT pregnancies present at Day 80, 5% had excessive fetal fluids at that time, and subsequently 42% were diagnosed with hydroallantois (by Day 150) and 25% became hydropic (by Day 210; Table I). Of all cases of hydroallantois, only a single recipient sustained pregnancy to term; a live calf was delivered by caesarean section 2 months after initial diagnosis.

There was different developmental potential for different bulls from which somatic nuclei were derived. Pregnancy rates were 38, 61, 24, and 15% at Day 60 and the calving rates (for those pregnant at Day 60) were 37.5, 50, 33, and 20% for pregnancies derived from bulls A, B,C (commercial group), and D (hand-made group) respectively.

Placentomes

There was significant difference among groups ($P<0.001$) and ages of gestation ($P<0.001$) with respect to placentome size. Overall, placentome length increased as pregnancy progressed, and was significantly greater in clones at all stages monitored (Day 80, $P<0.04$; Day 120, $P<0.003$; Day 150, $P<0.001$; Day 180, $P<0.03$; Day 210, $P<0.006$; and Day 240, $P<0.001$). This proved true for placentomes in all three 3 locations of measurement ($P< 0.0001$). In the control group, placentome length did not increase significantly after Day 180, as there were no significant differences between Days 180 and 210 ($P= 0.36$), nor between Days 210 and 240 ($P= 0.99$) resulting in flattened growth curve (Fig. 7A). In contrast, placentomes in the cloned group continued to increase in length during the last trimester of the pregnancy ($P<0.001$, Fig. 7B). In the control group, round or

oval placentomes were consistently present, whereas variant placentome abnormalities, as judged by shape and form, were detected ultrasonographically in cloned pregnancies.

The most frequent observation in placentomes from almost all clones was irregular shape and ragged edges, accompanied by hyper-echodensity (Fig. 4A); these characteristics became more prominent as pregnancy progressed. Structural differences were also detected in clones in comparison with other groups. Hyper-echodense borders, dark holes and streaks inside placentomes were observed, and the incidence in cloned pregnancies increased from Days 80 to 210 (5 to 50%, respectively), after which time 25% of affected pregnancies were lost (Table I). In both ultrasonographic monitoring and histological analysis of clone placentae, colonies of undeveloped and very small placentomes (length <1 cm) were observed (Fig. 5A). These mini-placentomes were detected in 33 and 55% of the NT pregnancies by Days 210 and 240 respectively (Table I), but were never detected in the control group.

Umbilical cord

There were effects of group ($P<0.001$) and age of gestation ($P<0.001$) on umbilical cord diameter. Umbilical diameter increased as pregnancy progressed, and was significantly larger in clones relative to controls at all stages of gestation except Day 80 (Day 80, $P<0.47$; Day 120, $P<0.0002$; Day 150, $P<0.02$; Day 180, $P<0.0001$; Day 210, $P<0.0007$; and Day 240, $P<0.04$). Thickening of the umbilical cord with hyper-echodense - spikes at the borders (Fig. 4C) was the major difference between clones and controls. This notwithstanding, no umbilical cord abnormalities could be detected by histopathology.

Amniotic and allantoic membranes

Morphometric observations of amniotic membranes from cloned and normal pregnancies are summarized in Table 2. There was significant effect of group at all stages of gestation (P<0001) on amniotic thickness. Overall, amniotic membrane was thicker (Fig. 4F) in cloned pregnancies, but the result was significant only in hand-made clones. Areas of alternating hypo- and hyper-echodense layers throughout the chorioallantoic compartment were the principal anomaly detected in clones. Macroscopically, the amniotic membrane appeared thicker with viscous discs diffusing through intercotyledonary spaces (Fig. 5A). These were seen in placentae from Day 150 and were usually accompanied by excessive allantoic fluid. Hydroallantois was the most frequent abnormality at day 150 (N=7) (Table I). The most obvious amniotic membrane abnormality was the appearance of the amniotic plaques (Fig.5C and 5D) observed in clones, on the amniotic membrane proximal to the fetus. These plaques were observed at Day 80 of gestation in 15% of NT pregnancies and decreased to 5% by Day 150 to completely disappear at the end of gestation (Table I). These were detected only rarely in control pregnancies. After Day 150, the most obvious change in the cloned placenta was thickening of the fetal membrane (Fig. 4F). This abnormality was detected in 5% of cloned cases at Day 80 and reached 55% by Day 240 (Table I).

Cloned pregnancies that were aborted between Days 150 and 180 (N=5) were analyzed retrospectively. In four of these, excessive allantoic fluid volume was detected by ultrasonography in earlier stages (Day 120), two had abnormal development of amniotic

plaques, (Day 80), while three had morphological anomalies on placentomes (e.g. hyper-echodense border and/or hypo- to hyper-echodense streaks) at Day 80 and 120.

Fetal fluid echodensity

This qualitative parameter was based on echodensity of the fetal fluids. There was an effect of group (P=0.02) and stage of gestation (P=0.007) for allantoic (Fig. 4C) but not amniotic fluid (P=0.06). The echodensity of the allantoic fluid increased with age of gestation. The conditions were not consistently present at all stages of pregnancy.

Histopathological observations

The most frequent change in placentomes from both groups of cloned pregnancies at all stages was the presence of necrotic cells. Their pattern of distribution changed as pregnancy progressed. At Days 150 and 180, necrotic cells were detected in discrete foci distributed throughout both fetal membranes; while they appeared extensively through the epithelium of the fetal membrane and caruncles at Day 240.

The presence of degenerated inflammatory cells (mostly neutrophils) and mononuclear cells (lymphocytes and macrophages) was obvious in clones (Fig. 6E and 6F). None were reported from control pregnancies. The presence of inflammatory cells was accompanied by hemorrhage or by thrombosis of large chorioallantoic vessels, particularly at Day 240 and parturition. In two cases, cotyledons detached from caruncles were observed and sero-hemorrhagic materials and necrotic cells filled the intercotyledonary

spaces. Cotyledon mineralization was less frequent, found only at Day 150. Presence of eosinophils around the vessels was observed in clones at parturition.

Edema of the chorioallantoic membrane was most obvious histopathological variation in cloned placentae, present only at Days 150 and 240. Decrease in epithelial surface thickness of both fetal membranes was also observed at Day 150; those cells disappeared almost completely by the time of birth (Fig. 6A). Some focal hemorrhages (Fig.6E and 6F) were seen, especially around the blood vessels at Day 240 and were accompanied by fibrosis with granulation tissues. Fibroblast proliferation, characterized by a range of responses from mild fibroplasias to fibrosis, was observed in amniotic membranes from Day 150 to parturition.

Discussion

Ultrasonography has proven to be an accurate and reliable tool to monitor the fetomaternal unit (Curran et al. 1986; Kastelic et al. 1988; Bertolini et al. 2002; Chavatte-Palmer, Heyman et al. 2002). The present study is the first ultrasonographic characterization of cloned placenta relative to normal counterpart, several differences between control (AI and ET) and cloned groups were evident.

Placentomes of NT pregnancies were larger and longer than their counterparts in control pregnancies. In all five cows with clones that had to be euthanized and were necropsied, the number of placentomes was lower than in a normal pregnancy (Schlafer et al. 2000), while but not as low as another report (Hill, Edwards et al. 2001). A reduced

number of placentomes was not found in any of the clones that survived to late pregnancy (Constant et al. 2006), supporting the hypothesis that only pregnancies with a sufficient number of placentomes can be maintained to term.

The difference in length between the NT and the control placentomes was present throughout the pregnancy (Days 80 to 240), not only between Days 120 and 180, as previously reported by Heyman (Heyman, Chavatte-Palmer et al. 2002). Placentomes in the cloned group continued to increase in length during the last trimester, contrasting with Constant et al., (2006) where larger placentomes were present only before Day 220 (Constant, Guillomot et al. 2006). In normal bovine pregnancy, placentome length increases linearly until Day 190 and then reaches a plateau (Laven et al. 2001). Furthermore, the differences in size between placentomes of cloned and control groups were consistent among locations sampled in the uterus. That larger placentomes of cloned pregnancies were associated with normal average and median birth weights (49 and 55 kg respectively) did not support the speculation that placentomegaly and the large offspring syndrome (LOS) are associated. In support of this view, Constant et al., (2006) showed that placental overgrowth was not a consequence of fetal overgrowth (Constant, Guillomot et al. 2006).

We observed irregular and hyper-echodense edges in placentomes of cloned pregnancies; most commonly during the second half of the last trimester. We did not observe obvious macroscopic changes in the thickness of the placentome in cloned pregnancies, as did Bertolini (Bertolini, Mason et al. 2002). Edema was frequent and appeared as a thick gelatinous layer surrounding the placentomes (Figure 5F). This

contrasts with other studies where edema did not occur in cloned pregnancies with significant frequency (Constant, Guillomot et al. 2006).

Pregnancies derived from clones studied at early stages have been reported to contain fewer placentomes than traditionally derived gestations (Hashizume, Ishiwata et al. 2002; Lee et al. 2004; Constant, Guillomot et al. 2006). Although differences in placentome development were evident ultrasonographically throughout pregnancy, placentome number could not be estimated by this technique. In the present study, small but well formed placentomes near fetus were detected from Day 210 (Figure 5A, Table I), without adventitious placentation-. These mini-placentomes appeared normal except for their size. In previous studies of cloned pregnancies, a many well developed mini-placentomes (< 1.0 cm) have been reported (Santos et al. 2006); they apparently had normal function, based on the presence of trophoblastic cells with PAS granules in the binucleate giant cells and intense subepithelial capillary organization. Further evidence from a stereological study of cloned pregnancy, suggested that the smaller placentomes were fully functional (Constant, Guillomot et al. 2006). These mini-placentomes may represent a compensatory mechanism to sustain insufficient placental exchange when there is a smaller number of placentomes and/or abnormal placental development. .

The rate of occurrence of hydroallantois of the study was 45%, consistent with previous reports of 33% (Hill, Roussel et al. 1999) and 50% (Heyman, Chavatte-Palmer et al. 2002). In contrast, in normal and IVP pregnancies the prevalence of hydroallantois is only approximately 0.01% and 0.5% respectively (Hasler et al. 1995). This uterine condition is the most common dropsical defect encountered in cattle (Peek 1997) and in

cloned pregnancies (Hill, Roussel et al. 1999; Chavatte-Palmer et al. 2000; Wells 2003). Hydroallantois has been classified by Farin as a Type II abnormal offspring syndrome condition, where there is no evidence or possibility of compensatory response by the fetus and/or the placenta (Farin, Piedrahita et al. 2006). This pathologic placental condition includes increased fetal fluid (determined by transabdominal ultrasonography) with difficulties in locating the fetus in the uterine horn (Heyman, Chavatte-Palmer et al. 2002). In most cases, an increased maximum fetal fluid thickness is evident prior to changes in the general condition or external appearance of the cow. We extend this finding by reporting the presence of edema characterized by areas of alternating hypo and hyper-echodense bands in the thickness of the utero-chorioallantoic layer and amniotic membrane. In severe cases of hydroallantois, pockets of fluid in the amniotic sacs and thickening of the chorioallantoic membrane described as multiple liquid-filled cysts were also observed in the present study. Some authors suggest that hydroallantois is associated with fetal renal failure (van Wagtendonk-de Leeuw et al. 1998). In our investigation, this condition was most commonly observed between Days 150 and 180 of cloned pregnancies and may account for pregnancy loss during this period. In spite of the high incidence of hydroallantois, the cause and progression of the condition during cloned pregnancy are not clear. There is evidence that it is associated with somatic cloning and is due to inappropriate expression of some imprinted genes (Kono 1998). Hydrops of the fetal membrane is usually associated with dramatic changes of the fetal fluid composition (Wintour et al. 1986). As an example, glucose is present in the allantoic fluid of IVP hydropic gestations on Day 180 (Bertolini et al. 2004).

No previous definitive ultrasonographic description of fetal fluids appears to exist. Our findings demonstrate that qualitative assessment of fetal fluids by ultrasonography has the potential to be a valuable tool for fetal well-being assessment, although taken alone it may not have strong predictive value (Adams-Brendimuehl et al. 1987). Based on the appearance of the fluid, a clear distinction between allantoic fluid (an-echodense) and amniotic fluid (hypo-echodense) containing particles (squamous epithelial cells from the epidermus and products of fetal deglutition) was observed in this study. Echodensity of the amniotic fluids changed with the stage of pregnancy with a maximum echodensity close to term. In ruminants, Jonker (2004) found particles repeatedly in fetal fluids in association with compromised fetuses (Jonker 2004). In contrast, other authors have shown that in normal human and equine pregnancy, particles are present (Adams-Brendimuehl and Pipers 1987; Manning 1987; Bucca et al. 2005) and Bucca et al., reference number confirmed this observation by systematically identification of free floating particles within both amniotic and allantoic fluid compartments from mid-gestation to term in equine pregnancy (Bucca, Forgarty et al. 2005). Abnormal echodensity of amniotic fluid has been associated with placentitis and septicemia, as well as with normal pregnancies in mares (Reef et al. 1996). The umbilical diameter was significantly larger in cloned pregnancies than in the control group and increased with stage of the pregnancy in both control and clones. Thickness of the amniotic membrane also increased with stage of gestation, and this membrane was also thicker in clones versus controls. The association between the thickening of the amniotic membrane and the presence of edema was corroborated by macroscopic and histological analyses. Edema of the fetal membranes was not homogenously distributed, making the

definitive diagnosis of increased thickness of the amniotic membrane more difficult. The presence of anomalies in other tissues is therefore required to confirm a diagnosis of abnormal pregnancy. If fetal membrane edema is associated with abnormal blood supply, changes in blood flow (velocity, diameter of vessels and resistance index) of uterine artery of pregnant horn may be assessed in future studies, allowing for increased understanding of the etiology of this phenomenon.

Before the appearance of the fetal membrane edema, amniotic plaques were visible. Plaques appeared at approximately Day 100, but disappeared after Day 150 when edema of the placento-uterine complex became evident. While we observed amniotic plaques exclusively in cloned pregnancies these plaques have been described in normal pregnancies during necropsy (Roberts 1986). It seems that they become larger and thicker in cloned pregnancy and then become detectable by ultrasonography. The significance of amniotic plaques is currently unknown as they were not associated with a negative outcome in the present study.

Histological analysis revealed important histological abnormalities in cloned placentae. The presence of inflammatory cells (lymphocytes and plasmocytes) and red blood cells in placentomes in cloned pregnancies suggested fetomaternal immunological reactions, consistent with the speculations of Hill et al. (2001) (Hill, Edwards et al. 2001). Fetal blood cell leakage from placental vessels or loss of trophoblastic cell integrity might explain the presence of these cells. Maternal immune cell infiltration within the villous space has been reported in human pregnancies (Kapur et al. 2004; Juliano et al. 2006). We further showed the presence of a dense layer of sub-epithelial necrotic tissues and edema

that correlated with epithelial disappearance and cotyledon detachment that we observed at last stage of NT pregnancies. Placentas of cloned pregnancies were characterized by chorionic and allantoic hypoplasia (Hill, Burghardt et al. 2000; DeSouza, King et al. 2001) and reduced epithelial height and cellularity (Peterson et al. 1998a; Peterson et al. 1998b; Hill, Burghardt et al. 2000). In contrasting studies, no major histological anomalies were found throughout cloned pregnancies (Ravelich, Shelling et al. 2004; Constant, Guillomot et al. 2006). These discrepancies may be the result of the large variation that exists among cell lines and laboratory techniques used to produce cloned animals.

In conclusion, we characterized placental changes through the 2nd and 3rd trimesters of pregnancy in bovine clones. A cluster of abnormalities, including amniotic plaques, the establishment of edema in the chorioallantoic membrane and the disappearance of the epithelium may lead to placental insufficiency and failure of pregnancy or low neonatal survival. We confirmed that placentomes of cloned pregnancies were larger than the control group at every stage of gestation and every location of the uterus. Also, based on our clinical observations, we conclude that the critical time when the fetal or mother life is threatened (between Days 150 and 180) could be predicted by following the pattern of placental changes. The problematic gestations could be ultrasonographically diagnosed by excessive allantoic fluid volume in early stages and accompanying placentome irregularities. As in early pregnancy, the large range of placental abnormalities described in late cloned pregnancy in cattle may account for a high proportion of embryonic and post-implantation fetal loss, and neonatal death.

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Tables and Figures

Day*	Group	% of fetal viability	Umb. cord diam. cm (LSM)	Abdomen circum. Cm (LSM)	Placentomes measurements		Morphological abnormalities in cloned placentas (no. of observations/total number of viable fetuses)	
					Length - cm (LSM)	Ave. No. of observation	Ultrasonography (no. of observations/total number of viable fetuses)	Histology
80	Control	100	1.05_a	221	1.94_a		1 - Hyper-echodense and distributed amniotic plaques (3/20) 2 - Fluid-filled blisters on amniotic membrane (1/20) 3 - Excessive allantoic fluid (1/20) 4 - Hypo-echodense holes on placentome (2/20) 5 - Hyper-echodense placentome border (1/20) 6 - Thick umbilical cord with hyper-echodense border (4/20)	
	Clone	100	1.19_a	224	2.63_b	4		
120	Control	100	1.85_a	224	3.35_a		1 - Hyper-echodense and distributed amniotic plaques (3/20) 2 - Fluid-filled blisters on amniotic membrane (2/20) 3 - Multilayer diffused edematous amniotic membrane (2/20) 4 - Excessive allantoic fluid (5/20) 5 - Hypo-echodense holes on placentome (2/20) 6 - Hyper-echodense placentome border (1/20) 7 - Thick umbilical cord with hyper-echodense border (4/20)	
	Clone	100	2.66_b	234	4.42_b	6		
150	Control	91.7	2.92_a	228	3.98_a		1 - Hyper-echodense and distributed amniotic plaques (1/19) 2 - Fluid-filled blisters on amniotic membrane (2/19) 3 - Multilayer diffused edematous amniotic membrane (4/19) 4 - Chorioallantoic edema (2/19) 5 - Excessive allantoic fluid (7/19) 6 - Excessive amniotic fluid (1/19) 7 - Hypo-echodense holes on placentome (1/19)	Placentomes : - Lesion and necrotic tissues - Macrophage infiltration localized on stroma - mineralization Chorion : - Edema - Necrotic cells on superficial stroma - thin epithelial surface Amniotic membrane : - Fibrosis
	Clone	95	3.45_b	248	5.57_b	6		
180	Control	91.7	3.03_a	254	5.25_a		1 - Fluid-filled blisters on amniotic membrane (2/14) 2 - Multilayer diffused edematous amniotic membrane (2/14) 3 - Excessive allantoic fluid (3/14) 4 - Chorioallantoic edema (2/14)	Placentomes : - Necrotic epitehlai cells - Focal inflammatory cells regions Amniotic membrane : - Amniotic plaques
	Clone	70	4.12_b	253	6.13_b	6		
210	Control	91.7	3.84_a	251	5.41_a		1 - Fluid-filled blisters on amniotic membrane (2/12) 2 - Multilayer diffused edematous amniotic membrane (2/12) 3 - Chorioallantoic edema (2/12) 4 - Excessive allantoic fluid (3/12) 5 - Hyper-echodense placentome border (6/12) 6 - Miniplacentomes (4/12) 7 - Thick umbilical cord with hyper-echodense border (1/12)	
	Clone	60	4.71_b	261	6.47_b	6		
240	Control	91.7	4.18_a	256	5.82_a	6	1 - Fluid-filled blisters on amniotic membrane (1/9) 2 - Multilayer diffused edematous amniotic membrane (4/9) 3 - Chorioallantoic edema (1/9) 4 - Hyper-echodense placentome border (2/9) 5 - Miniplacentomes (5/9)	Placentomes : - Excessive trophoblastic necrotic cells - Degenerated inflammatory cells - Presence of mononucleates - Thrombosis - Detached cotyledons from caruncles (Parturition) Chorion : - Diffused edema on degenerated stroma - Hemorrhagic plaques - Spread fibrosis - Absence of epithelial surface - Mononucleates infiltration Amniotic membrane : - Amniotic plaques - Mononucleates infiltration - Absence of epithelium - Fibrosis
	Clone	45	4.99_b	267	8.39_b			

Table I - Morphology in control vs. clone bovine pregnancies

Continuous data are least square means. Significant differences are shown by ‘a’ and ‘b’ (*P<0.05*). * - Day 80 was assumed as reference point

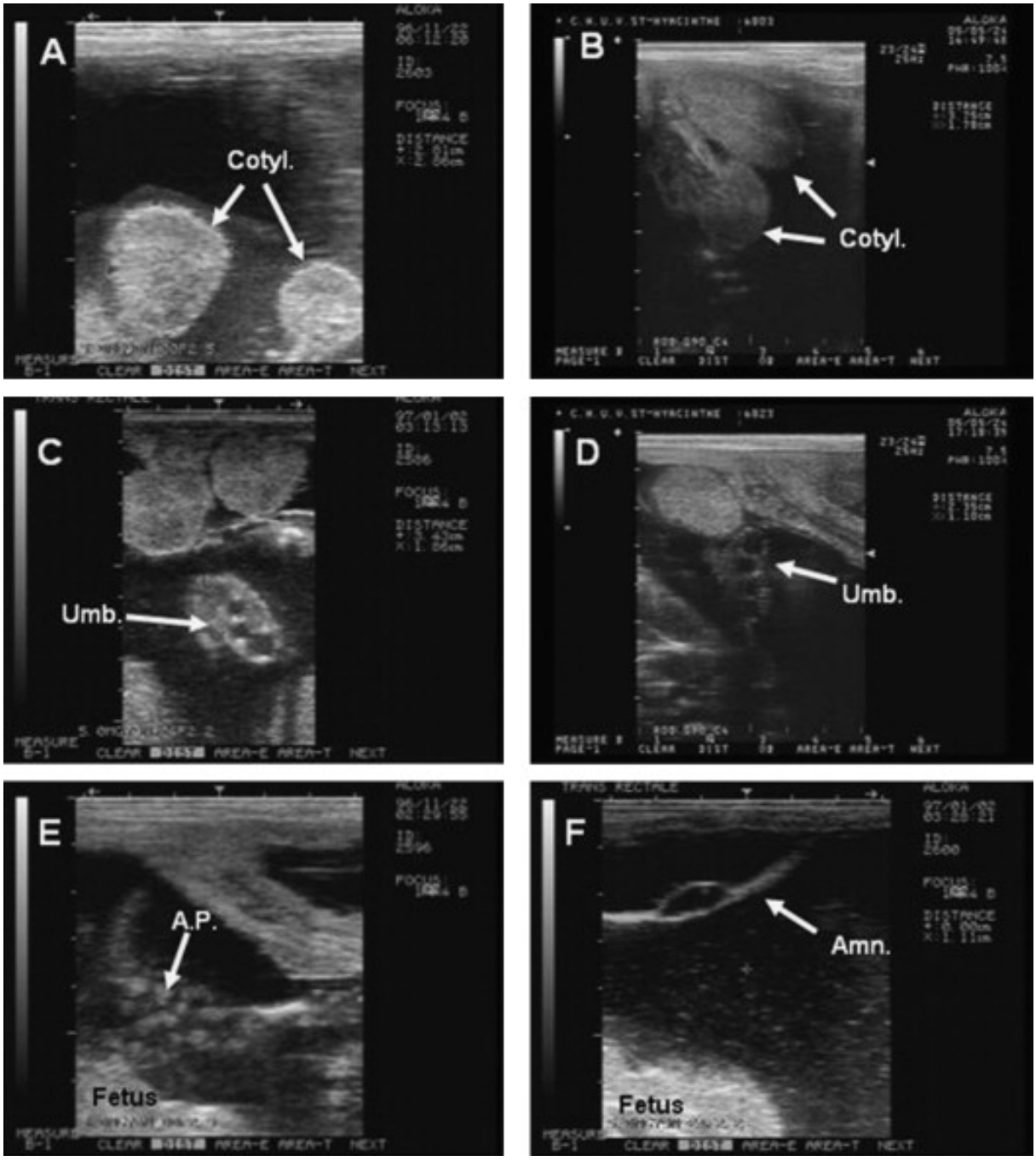
A
Cotyl.
B
Cotyl.
C
Umb.
D
Umb.
E
A.P.
Fetus
F
Amn.
Fetus

Figure 4 - Ultrasonography of fetal membranes during gestation in both cloned and control pregnancies.

At day 80, hyper-echodense borders and irregular structures of cotyledons were common in placenta of cloned (NT) fetuses (A) in comparison to controls (B). Edematous umbilical cord with hyper-echodense spikes around was present in NT (C) versus AI pregnancies (D) at day 120. Amniotic membrane anomalies were also observed in cloned pregnancies, such as abnormal appearance of amniotic plaques near the fetus (Day 80) (E) and vesicles on this membrane (Day 120) (F). Abbreviations: Cotyl. Cotyledon; Umb. Umbilical cord; A.P. Amniotic plaques; Amn. Amniotic membrane.

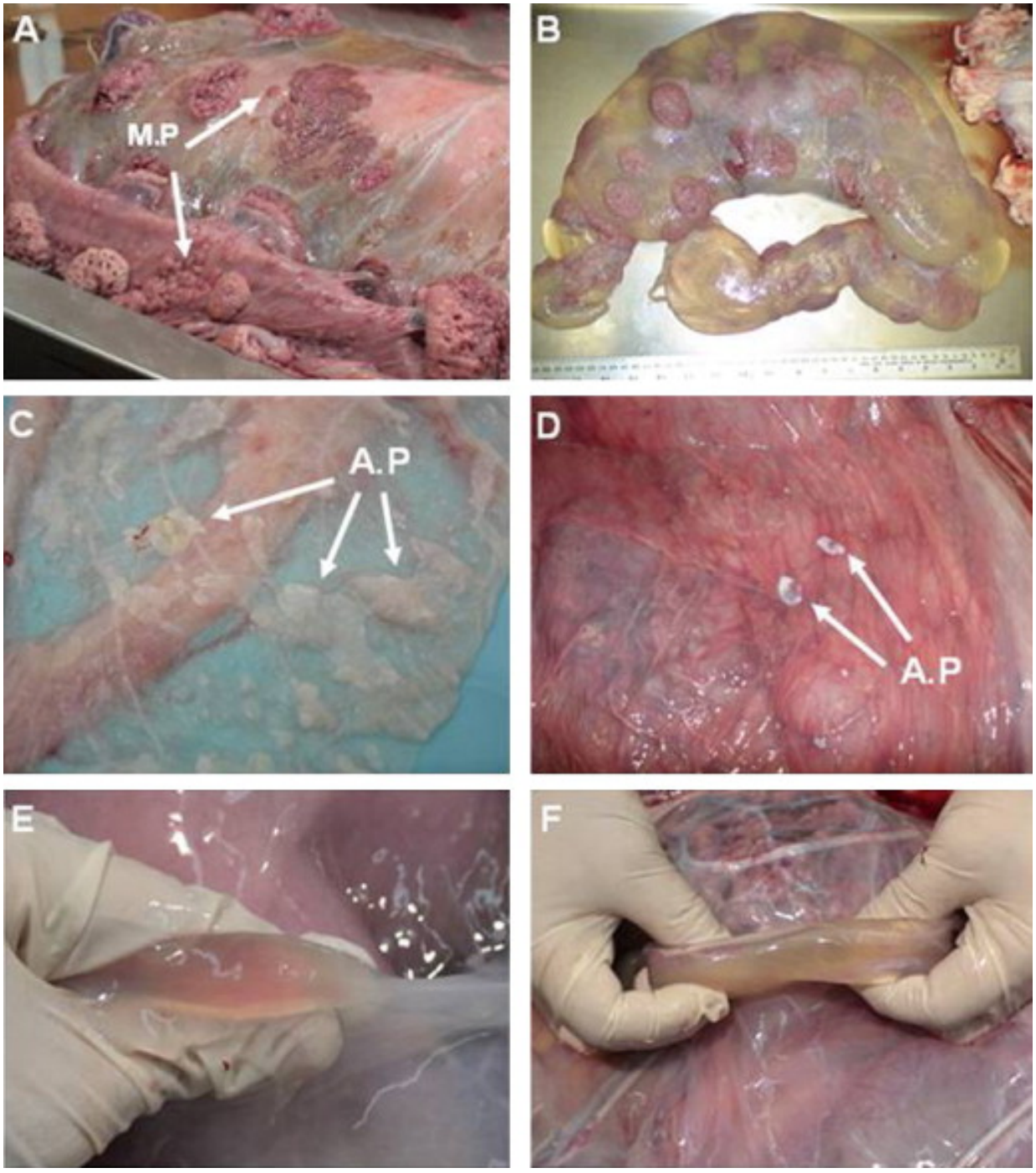
A
M.P
B
C
A.P
D
A.P
E
F

Figure 5 - Macroscopic observations of collected tissues in necropsy

Mini-placentomes (M.P) an apparent compensation strategy for the needs of NT fetus (Day 120) (A) were not observed in normal gestation (Day 120) (B). Abnormal formation of amniotic plaques (A.P.) in NTs (Day 240) (C) compared to controls (Day 240) (D) was observed on this membrane. Focal edema on both amniotic (Day 120) (E) and chorioallantoic (Day 150) (F) membranes in cloned pregnancies was commonly observed in NTs.

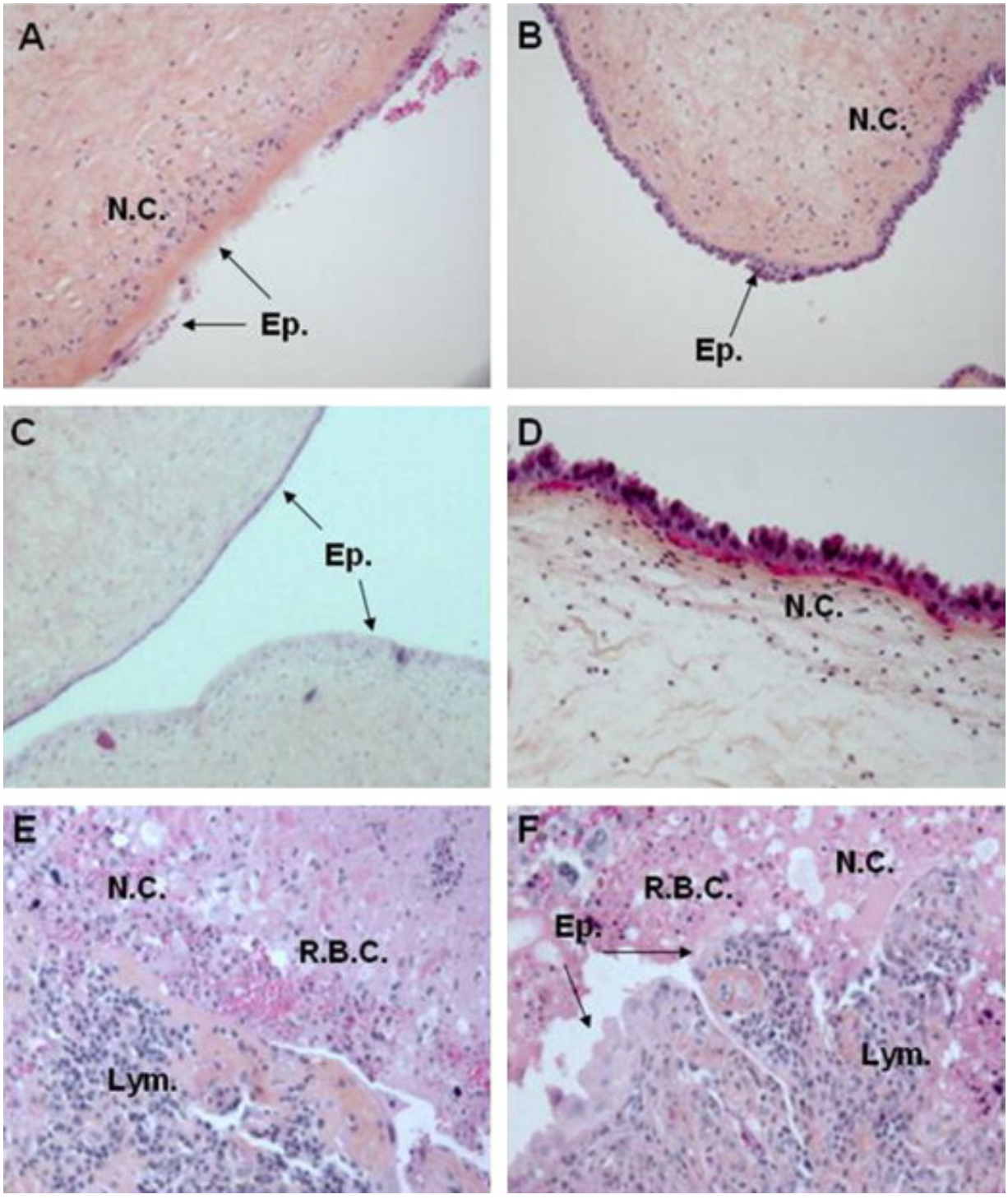
A
N.C.
Ep.
B
N.C.
Ep.
C
Ep.
D
N.C.
E
N.C.
R.B.C.
Lym.
F
N.C.
R.B.C.
Ep.
Lym.

Figure 6 - Light micrograph of fetal tissues

Amniotic membrane (A,B), chorioallantoic membrane (C,D) and cotyledon (E,F) from cloned pregnancies collected at parturition. The serum leakage and red blood cell (R.B.C) between the maternal and fetal tissues in cotyledons (E,F) was accompanied by degenerated inflammatory (Lymphocytes (Lym.)) and necrotic cells (N.C.). Localized disappearance of epithelium (Ep.) in all three regions is also indicated by arrows.

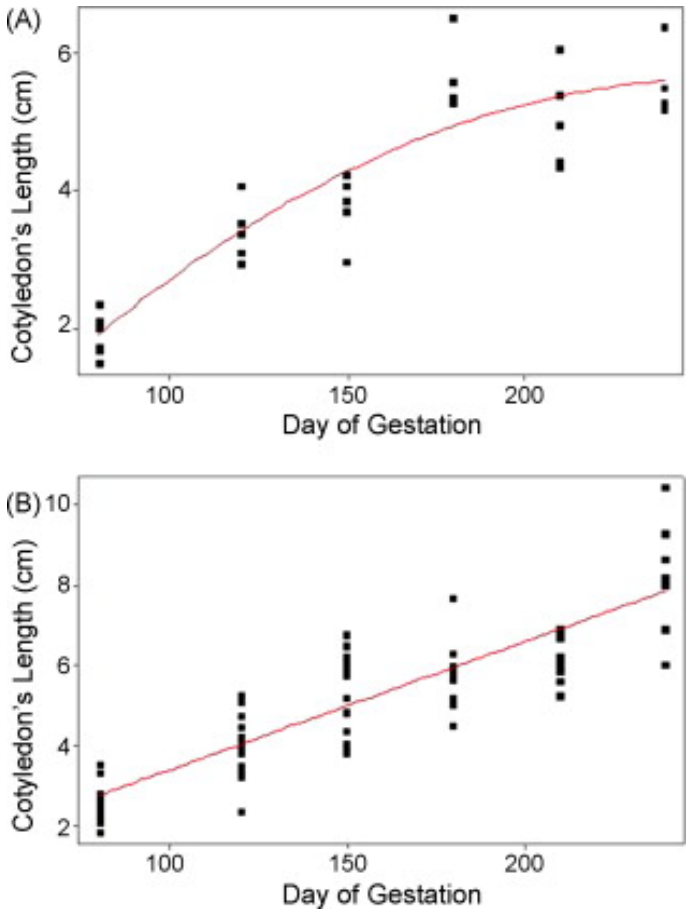

Figure 7 - Comparison of polynomial regression curves of cotyledon length

Comparison of polynomial regression curves of cotyledon length between two groups of pregnancies showed that in controls the growth curve tends to be flattened after Day 180 where in clones the cotyledons seem to continue their growth, through the final stage of gestation.

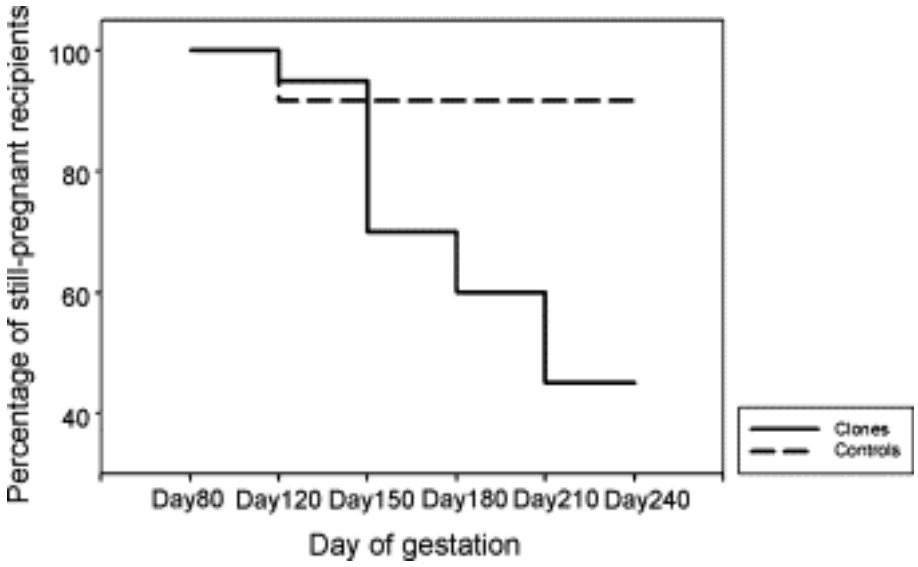

Figure 8 - The comparison of percentage of persisting pregnancies

Comparison of percentage of persisting pregnancies between two groups of animals bearing cloned fetuses reveals high rate of fetal loss during second and third trimester of NT gestations.

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ARTICLE III: Endocrine Profiles of Somatic Nuclear Transfer-Derived Pregnancies in Dairy Cattle

Status: Submitted

Endocrine Profiles of Somatic Nuclear Transfer-Derived Pregnancies in Dairy Cattle

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Abstract

In cattle, several hormones and proteins are necessary for maintenance of a normal pregnancy that will result in a viable calf. In cloned animals, high incidence of pregnancy loss and prenatal morbidity and mortality occur and are believed to be due to placental insufficiency. Deviation from the normal cascade or expected profile of reproductive hormones and proteins may be associated with impairment of somatic nuclear transfer-derived pregnancies and the high rate of fetal loss. The objectives of this study were to characterize the maternal serum concentrations of pregnancy-specific protein B (PSPB), progesterone (P4), estrone sulphate (E1S), and estradiol (E2) during the last two thirds of cloned bovine pregnancies and to evaluate their association with the gestational abnormalities. A group of cows with cloned fetuses, produced by either commercial (n=16) or zona-free (n=4) cloning techniques were compared to pregnant animals derived from traditional embryo transfer (n=6) or artificial insemination (AI, n=6), at various stages of gestation (Days 80, 120, 150, 180, 210, and 240; Day 0 = estrus). Blood samples were collected from the tail vein into vacuum test tubes and placental changes and fetal wellbeing were monitored with ultrasonography throughout the pregnancies. Only eight (40%) cloned pregnancies reached term and seven calves (35%) survived. It was possible to demonstrate a deviating endocrine profile in somatic nuclear transfer-derived pregnancies. At day 80, P4 concentration was significantly lower ($P< 0.0001$) in NT recipients than the control groups. Mean estrone sulphate concentrations did not vary significantly between NT and control groups. At day 150, PSPB concentrations were elevated ($P<0.002$) in NT

cows. Estradiol concentration was higher in NT recipients throughout the study period compared to control cows. We infer that these hormonal changes along with morphological anomalies of placenta result in compromised fetal development.

Introduction

Transfer of bovine embryos cloned by nuclear transfer (NT) has been associated with high incidence of pregnancy loss and prenatal morbidity and mortality (Hashizume et al. 2002). Specific problems, such as abortion, hydroallantois, abnormally long gestation, higher incidence of dystocia and large offspring syndrome (LOS), have been previously reported (Kohan-Ghadr et al. 2008). Although the specific fetal pathology varies, the low efficiency of cloning in cattle is caused, at least in part, by atypical placentation with attendant alterations in morphology and gene expression, leading to metabolic or other anomalies (Hill et al. 1999; Farin et al. 2006; Kohan-Ghadr et al. 2008). As steroid hormones and reproductive proteins play a key role in pregnancy recognition and maintenance, this cluster of placental abnormalities (Sakai et al. 2005) in cloned animals is expected to be associated with abnormal endocrine profiles. .

Progesterone (P4) plays a key role in the reproductive events associated with pregnancy. In cows by Day 17 post-conception, P4 from the corpus luteum is associated with conceptus elongation, production of interferon-τ, placental development and higher pregnancy rates (McNeill et al. 2006). Progesterone decreases myometrial contractility, and is known to stimulate the glands of the endometrium to proliferate and to secrete the histotroph or

uterine milk necessary to nourish the embryo before establishment of the placenta. The positive effect of P4 on the advancement in conceptus elongation is most likely associated with changes in gene expression, endometrial secretion and uterine environment (Clemente et al. 2009; Forde et al. 2009). While P4 is produced by the corpus luteum in early pregnancy, its role in maintenance of pregnancy varies among species. In cows, the corpus luteum produces P4 throughout gestation. However, the placenta generates sufficient P4 to maintain pregnancy from Day 200 of gestation (Estergreen et al. 1967). A few days prior to parturition, there is a sharp decline in plasma P4 (Zhang et al. 1999). Abnormal P4 levels in recipients carrying NT or IVF fetuses are believed to be associated with an increase in birth weight (Walker et al. 1996) and abnormal growth of organs and skeleton (Farin et al. 1995).

Estrone sulphate (E_1S) is the major conjugated estrogen in the pregnant maternal circulation. It is secreted from the cotyledonary portion of the bovine placentome and measurement of concentrations in peripheral circulation has been employed to assess fetal-placental-maternal wellbeing and the sufficiency of the placenta (Dobson et al. 1993; Echternkamp 1993). Estrone sulphate can be detected in peripheral maternal blood between days 70 and 100 of gestation and concentrations increase throughout gestation (Zhang et al. 1999; Kindhal et al. 2002). In cows, the specific biological roles of placental estrogens are not well understood (Hoffmann et al. 2002). It is known that insufficient production of E_1S and delayed regression of corpora lutea are associated with dystocia in dairy cows (Zhang et al. 1999). Twin pregnancies display higher levels of E_1S compared to singletons (Worsfold et al. 1989). E1S concentration has been shown to be decreased in cases of

dystocia in dairy cattle (Zhang et al. 1999). E_1S levels were also positively correlated with calf birth weight, weights of cotyledons, of inter-cotyledonary membranes and of the total placenta from day 210 of gestation, and with the neonatal viability (Zhang et al. 1999).

In addition to steroid hormones, placenta of ruminants secretes pregnancy-specific protein B (PSPB), one of a family of pregnancy-associated glycoproteins (PAGs) (Butler et al. 1982; Zoli et al. 1991). PSPB is synthesized by the trophoblastic binucleate cells and is detectable in the form of increasing circulating concentrations from Day 28 to 270 of bovine gestation (Zoli et al. 1992). During the postpartum period, the PAG levels progressively decrease to undetectable concentrations by approximately 100 days after calving (Zoli et al. 1992). Concentrations of PSPB are altered by in vitro fertilization, in nuclear transfer derived pregnancies and in uterine diseases (bacterial infection) that cause embryonic death (Semambo et al. 1992; Patel et al. 1997; Szenci et al. 1998). Higher concentrations of PAG are present in both recipient heifers (Ectors et al. 1995) and cows (Chavatte-Palmer et al. 2006) carrying cloned conceptuses, compared to those carrying fetuses from in vitro produced embryos. The pattern of PSPB concentration varies in the recipient's peripheral circulation and this parameter has been used as a tool to investigate placental function and fetal wellbeing (Patel et al. 1995; Patel et al. 1995; Patel et al. 1997; Becker et al. 1999). The specific function of PSPB remains largely unknown, but plasma concentrations have been correlated with the stage of gestation (Zoli et al. 1992; Green et al. 2005), the number of foetus (Dobson et al. 1993; Echternkamp et al. 2006), foetal sex (Zoli et al. 1992), and early foetal loss (Lopez-Gatius et al. 2007). Angiogenesis (Austin et al. 1999) and immunosuppressive (Wooding et al. 2005; Lopez-Gatius et al. 2007)

properties have been attributed to PAGs, due to their capacity to stimulate vascularisation in early placentation and abrogate maternal rejection of the foetus respectively.

As most literature has focused on dysfunction at the time of implantation in nuclear transfer surrogate dams, the present longitudinal study was undertaken to assess the endocrine profiles of P4, E1S, E2, and PSPB during the last two trimesters of cloned bovine pregnancies. Comparisons are made to pregnancies derived from artificial insemination and embryo transfer, and the hormones concentrations have been investigated in the context of assessment of placental abnormality and fetal wellbeing.

Materials and methods

Animals and embryo production

Animals and embryo production protocols have been described previously (Kohan-Ghadr et al. 2008). Briefly, embryo recipients were Holstein heifers, 15 to 18 months old, average body condition score of 3.5 (scale, 1-5) (Heinrichs 1985) . Estrus was synchronized using two injections of prostaglandin F2α at 10 day intervals (Seguin et al. 1989). Following detection of estrus, heifers were inseminated (n=6), or they received a frozen-thawed (n=6) or a fresh cloned embryo (n=20). Cloned embryos were produced either in university laboratory using zona-free cloning technique (Vajta et al. 2006) or in a private company through commercial cloning protocol. The reconstituted embryos were cultured for 7 days before transfer to recipients. All procedures conformed to the National

Guidelines for Care and Use of Laboratory Animal and were approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine of the University of Montreal, Saint-Hyacinthe.

Fetal ultrasonography assessment

Pregnancy was confirmed at Days 30 and 60 and experimental monitoring began at Day 80. All recipients were observed daily during gestation and ultrasound analysis was carried out at each stage (Days 80, 120, 150, 180 and 240) by a transabdominal and transrectal ultrasonography using a 3.50 and 5.00 MHz probe respectively (ALOKA 900 , Alliance Medical Inc., Montreal, Quebec, Canada). The viability of the fetus and ultrasonographic aspects of placental development were monitored and recorded with a digital video camera (Sony, DCR-HC90 Handycam, Tokyo, Japan). Fetal viability was assessed by fetal heartbeat and movement. Placentome size, shape and appearance, presence or absence of edema and any other abnormalities in fetal membranes were recorded.

Blood samples collection and serum extraction

Blood samples were collected from the coccygeal vein using 20-gauge needles at days 80, 120, 150, 210 and 240 of gestation into vacuum 10 ml glass EDTA tubes. Immediately after collection, the plasma was obtained by centrifugation (1500 x g) for 15

minutes and the supernatants were stored at -20 °C in 1 ml vacuum glass tubes until the assay was performed.

Progesterone and estradiol 17-β (E2) RIA

Concentrations of P4 and E2 were measured in serum by standard radioimmunoassay protocol following extraction using diethyl-ether. For the P4 assay, recovery rates were between 90% and 95%. The anti-progesterone-11-BSA antibody was kindly provided by Dr. Gordon Niswender (Colorado State University, Fort Collins CO). The sensitivity of the assay was 62.50 pg/ml of serum. The intra-assay coefficient of variation, calculated between replicates was 3.58%. The interassay value, calculated from 5 replicated samples present in all assays, was 13.36%. The antibody used for E2 detection was 'LMS157', raised in sheep and provided kindly by Dr. Lee Sanford, formally of McGill University (Montreal QC). It has approximately 15% cross-reactivity with estrone, and negligible cross-reactions with other steroids (Sanford 1987). The intra- and inter-assay coefficients of variation were 5.09% and 3.11%, respectively.

PSPB RIA

Bovine PSPB was determined by radioimmunoassay according to procedures described by Sasser et al. (Sasser et al. 1986) (BioTracking, Moscow, ID, USA). Pure bovine PSPB (R-37) was used as tracer and standard. Antibody RGS-38-1 against bPSPB

(bovine-PSPB) from rabbit was used for binding at a final dilution of 1:145,000. The sensitivity of the assay was 0.50 ng/ml.

Estrone sulfate (E_1S) ELISA

E_1S concentration of plasma samples were measured using double antibody ELISA direct method without extraction (Isobe et al. 2002). Anti-estrone-3-glucuronide-BSA IgG (COSMO BIO Co., Tokyo, Japan) was employed as first antibody. The second antibody was HRP-labelled estrone-carboxymethylether (COSMO BIO Co., Tokyo, Japan). For development TMP (3,3',5,5'-Tetramethylbenzidine, Sigma, USA) was added as substrate solution and the optical density was measured at 492 nm in a microplate reader. The intra-assay and inter-well coefficient of variation was 8.70% and inter-assay value was 10.8%.

Experimental design and statistical analysis

To study effects of gestation (AI vs. embryo transfer vs. cloned embryo) and stage of gestation on peripheral hormone concentrations, we used a repeated measurement linear model with group of gestations as between subject factor and day of pregnancy as within-subject factor. For E_1S assay values, logarithmic transformation was applied to normalize the distributions. Other data were analyzed without transformation. Because of the large number of contrasts, we used the Bonferroni sequential correction procedure to ensure that the overall type I error rate was not above the nominal level of statistical significance.

Another set of analysis was run to study the possible difference in hormone concentration between NT recipients who aborted at each stage (Day 180, 210 and 240) and those that pass to the next one (alive NT). As for the first part of the analysis, the same linear model was used to compare the 2 groups. The concentration of hormones is shown as least square means (LSM±SEM). The level of significance in this study was $P<0.05$. Relationship between hormones and size of placentomes was evaluated using a mixed linear model with the animal as a random factor to control for multiple measurements of each individual.

Results

Of 20 NT pregnancies confirmed at Day 80, only eight (40%) reached Day 240. The 12 pregnancies that did not proceed to term were therapeutically aborted for humane reasons (at day 150 (N=1), 210 (N=1) and 240 (N=2)), caesarean section (at day 210 (N=1), and 240 (N=2)) or abortion (at day 150 (N=3), 180 (N=1) and 210 (N=1)). The placentome length increased as pregnancy progressed, and was significantly greater in clones at all stages monitored (Day 80, $P<0.04$; Day 120, $P<0.003$; Day 150, $P<0.001$; Day 180, $P<0.03$; Day 210, $P<0.006$; and Day 240, $P<0.001$). In the control group, placentome length did not increase significantly after Day 180 ($P>0.05$). Mini-placentomes were detected in 33 and 55% of the NT pregnancies by Days 210 and 240 respectively, but were never detected in the control group. Hydroallantois was the most frequent abnormality at day 150 (N=7). This abnormality was detected in 5% of cloned cases at Day 80 and reached 55% by Day 240 (Kohan-Ghadr et al. 2008).

Statistical analysis revealed significant effects of group of gestation (NT vs. control) and stage of pregnancy on progesterone concentration. Even though pattern changes in serum P4 concentration were measured in the second trimester, similarities were evident between the two groups during the third trimester of gestation (Fig. 9A). At day 80, mean of P4 level for the controls was significantly higher ($P<0.0001$) than NT recipients with 5.94 ± 0.45 ng/mL and 3.24 ± 0.32 ng/mL respectively. During the second third of gestation, P4 concentration of controls decreased to reach 3.34 ± 0.58 ng/mL at day 180. During the same period, the NT recipients that had considerably lower P4 concentration kept approximately constant levels of P4 up to day 180 (3.32 ± 0.37 ng/mL). During the final third of pregnancy, both groups showed a similar P4 profile with an increase at day 210 (control and NT recipients with 4.84 ± 0.46 ng/mL and 4.20 ± 0.41 ng/mL respectively) followed by a decline (3.92 ± 0.48 ng/mL and 3.22 ± 0.48 ng/mL, respectively) at day 240 reaching similar levels of day 180. In control group, the estimation indicated a strong trend toward an inverse relationship between P4 concentration and the size of the placentomes. However, the trend was significantly positive in NT gestations (Table II).

Throughout gestation, E1S increased significantly ($P<0.001$) at a steady rate in both groups with no significant difference between them (Fig. 9B). Means of E1S concentrations (± SEM) varied from 0.99 ± 0.67 ng/mL to 7.82 ± 1.40 ng/mL in control and from 1.18 ± 0.43 ng/mL to 6.92 ± 1.17 ng/mL in NT recipients. Although in NT pregnant cows, E1S was lower at Day 80 (1.19 ± 0.77 ng/mL) compared to controls (2.87 ± 1.51 ng/mL). The

relationship between E1S level and size of placentomes were significantly positive in both groups of gestation (Table II).

Contrary to E1S, the E2 concentration profile differed between the control and NT groups throughout gestation. In controls, E2 concentration was approximately constant with a mean of 47.80 ± 2.60 pg/mL during the second trimester (Day 80-Day 180). During the third trimester, it increased rapidly to about two fold (78.60 ± 10.40 pg/mL) at Day 240. In NT group, E2 concentration was already higher than the control group at day 80 (71.00 ± 7.20 pg/mL) and continued to increase at day 240 (109.20 ± 10.20 pg/mL) with a slight decrease at day 210 (92.40 ± 8.90 pg/mL). Like E1S, E2 was related to the placentomes size in both groups (Table II).

During monitoring (day 80 to day 240) we observed that PSPB concentration varied from 3.10 ±10.14 to 65.47±10.95 ng/mL and from 6.08±7.46 to 84.83±10.65 ng/ml in control and NT pregnancies, respectively. Statistical analysis revealed that overall, there is no significant difference in PSPB concentration between control and NT pregnancies however, both groups showed a significant elevation throughout pregnancy (Fig. 9D). The only exception is at day 150, when NT pregnancies showed a significantly higher concentration of PSPB compared to controls. A positive and significant relationship of PSPB and placentome size was found in both groups (Table II).

Discussion

The present study provides for the first time insight on hormonal changes, placenta abnormalities and gestation losses in somatic nuclear transfer-derived pregnancies in dairy cattle during the second and third trimesters of gestation. The main significant findings of this study are the higher level of E2 in NT pregnancies and the potential use of the hormonal profile as an indicator to predict abnormal gestation.

Values of serum E2 in the control group of cows appeared to be higher than those reported by Aldelakoun et al. (Adelakoun et al. 1978) and Gabai et al. (Gabai et al. 2004). However, in the present, as in the previous studies, E2 concentration progressively increased between day 80 and day 240 of gestation. The effect of stage of pregnancy significantly affected the circulating levels of E2 in normal pregnant animals with the most significant increase appearing after day 250 of the gestation (Gabai et al. 2004). Estradiol concentrations were higher in NT recipients than in control animals during the whole monitoring period (day 80 to 240). However, the statistical significance was only measured from Day 120 to Day 180 of the gestation. The NT recipients that lost gestations between day 180 and 210 showed a decrease in the mean concentration of E2 at day 180 compared to continuing NT pregnancies. Of all NT pregnancies, neonatal mortality (> Day 240) occurred in cows (N=3) with a lower E2 serum concentration at earlier stages. Several factors can influence concentrations of serum E2 (placental mass, blood flow, compartmental distribution, fetal factors) in NT pregnancies. The present longitudinal study with follow-up during most of the pregnancy period allows a better assessment of the

variations. In cattle, trophoblastic cells express the enzyme cytochrome P450c17, a key enzyme in the steroidogenic pathway for the formation of androgenic precursors for estrogen synthesis. Therefore, trophoblastic cells are potentially able to synthesize androgens and estrogens (Conley et al. 1992). The overall higher concentration of E2 could be explained by the notion that changes in steroidogenic activity of the placenta result largely from changes in tissue mass (Conley et al. 1990). Placentomes of NT pregnancies are larger and longer than their counterparts in control pregnancies and they continue to increase in length during the last trimester while, over the same period, they reach a plateau in normal gestations (Laven et al. 2001). Furthermore, the most significant increase in placentome size was measured in the last trimester and Day 150 stage revealed the highest frequency (n=7) of hydroallantois. The higher levels of E2 could also be a consequence of a higher production of fetal glucocorticoids. Both steroidogenic enzyme expression and concentrations of glucocorticoids in bovine fetal adrenal glands during the first trimester (Lund et al. 1988) and late in gestation (Thorburn et al. 1979; Matamoros et al. 1994) have been demonstrated. Chavatte-Palmer et al. (Chavatte-Palmer et al. 2002) showed that at term cloned fetuses were not premature based on normal response to ACTH. In an in vitro experiment, it was shown that cortisol does not affect E2 production in mono and binucleate cells collected from mid-gestation uteri (Matamoros et al. 1994). An increase number of mono- or binucleate trophoblastic cells may explain a greater synthesis of estrogens (E1S and E2) (Gross et al. 1988; Matamoros et al. 1994). Ravelich et al. (2004) reported a significant increase of binucleate cells in somatic cell nuclear transfer cloned placenta (Ravelich et al. 2004). In contrast, Arnold et al. (2006), using similar cell

characteristics to the present study, showed a reduced number of binucleate cells in cloned placenta compared to the AI and IVF animals (Arnold et al. 2006). This contradiction may suggest other placental cell types (eg. uninucleate cells) as source of E2 (Matamoros et al. 1994) or may represent an indirect effect of the ovarian steroidogenesis.

Estradiol modulates several vascular functions including inflammation, wound healing and angiogenesis (Cid et al. 2002) and more specifically, cell functions such as migration and proliferation (Johns et al. 1996). This action is possible by affecting the intercellular adhesion through the disconnection of adhesion junction complexes from the cytoskeleton by a membrane-associated signalling pathway (Groten et al. 2005). The large concentration of E2 in NT pregnancies could exacerbate the edema of the placenta that is common to the pathophysiology of large number NT gestations. Macroscopically, the amniotic membrane appeared thicker with viscous and edematous appearance diffusing through intercotyledonary spaces (Kohan-Ghadr et al. 2008). Edema of the chorioallantoic membrane was macroscopically and histologically obvious in cloned placenta. The excessive amount of E2 could change extracellular fluid distribution across compartments where distribution is controlled by osmotic, hydrostatic, and colloid osmotic pressure (Greenleaf 1990). These Starling forces dictate fluid movement between the vascular space and the interstitium. Hydroallantois was the most frequent abnormality at Day 150 of pregnancies in the fetuses in this study (Kohan-Ghadr et al. 2008). The presence of inflammatory cells accompanied by hemorrhage or by thrombosis of chorioallanotic vessels and detachment of cotyledons from caruncles could be associated with events such as disconnection of cell-cell adhesion. Interestingly, the recipients who gave birth to abnormal

or dead NT offspring after a normal gestation length showed less E2 concentration from the mid-gestation compared to the recipients of normal NT (Fig. 10). The high E2 concentration in maternal blood may imply a local excessive level of E2 in the placental tissues. This high level of E2 may induce changes in the tissue interface between the mother and the fetus and represents a potentially risk factor for fetal and neonatal abnormalities. The fact that estrogen receptors have been identified in differing carunclar cell types (Schuler et al. 2002) suggests there may be an important local role of E2. The level of E2 reported in the present study contrasts with previous studies that showed cows carrying clone fetuses that often do not show appropriate preparation for birth at the estimated date of parturition (Wells et al. 1999).

E2 has a dose and time dependent effect on the events during gestation such as angiogenesis (Ma et al. 2001), fetal energy source availability from adipose tissue (Pallottini et al. 2008), insulin resistance (Gonzalez et al. 2000) and immune suppression by regulation of T cell proliferation (Prieto et al. 2006). In humans, there is evidence suggesting a potential role for estrogens in mediating placental trophoblast growth and development (Jiang et al. 1997; Jeschke et al. 2007). So, extended exposure to a high E2 concentration could compromise fetal wellbeing and alter adaptation and survival ability of the fetus in a changed uterine environment. As higher concentrations of E2 are associated with pregnancy losses and abnormal placentation, the results of the present study suggest that the deviation of E2 concentration in circulation of NT recipients from the mean of normal NT pregnancies (with live NT calves) could be used as a predictor of problematic

pregnancies. E2 concentration in circulation in NT recipients may serve as a new clinical standard and as the basis for future studies.

Estrone sulphate (E1S) is principally produced by the placenta during the gestation in cattle (Hirako et al. 2002). The present results showed a gradual increase in E1S plasma concentration between Day 120 to 240 of gestation. The mean plasma E1S concentration at each stage of the study was not different between the two groups except at early stage (day 80) when the NT group had lower concentrations. This observation is in agreement with the previous report of a comparative study on E1S profiling of bovine recipients of NT fetus during gestation (Shah et al. 2007). In normal cows, Patel et al. reported low plasma E1S concentrations (< 5 pg/ml) until day 60 (Patel et al. 1999). The majority of established NT pregnancies in cattle are lost between day 30 to 90 of gestation, in association with poorly developed placentomes (Hashizume et al. 2002). Somatic nuclear transfer-derived placentomes have abnormal shapes and are present in reduced numbers (Miglino et al. 2007). In the present study, the lower levels of E1S at day 80 might be associated with poor placental development in NT gestations in comparison to control cows, while pregnancies that placenta were sufficiently developed to maintain their pregnancy and to synthesize E1S. Whether the perturbation in maternal E1S concentration is a cause for placental malformation or it is a consequence of placental dysfunction requires to be investigated. Although the large variance compromised the significance level of the present analysis, the present study showed that at all stages, NT pregnancies have similar E1S concentration to controls. The relation between E1S concentration in maternal circulation and associated problematic early pregnancies with placental malformation remains controversial. In the

present study, no information was available before Day 80. Hormone E1S level was correlated with calf birth weight, weight of cotyledons, intercotyledonary membranes and total placenta from day 210 gestation (Zhang et al. 1999). In the present study, E1S profile between live and aborted NT recipients was different for recipients which lost their fetuses between Day180 to 210 and those that aborted between Day 210 to 240. Interestingly, in both groups, an increase in E1S concentration coincided with a decrease in E2 level, perhaps an indication of changing balance between active and inactive form of estrogens. Further studies are needed to confirm the use of E1S profile in NT pregnancies as a predictor for problematic pregnancies.

At day 80, the P4 concentration of control cows was significantly higher compared to NT recipients. During the first trimester of pregnancy in dairy cattle, Ayad et al. (2007) reported a P4 concentration of 7.10 ± 3.60 ng/ml at day 80 (Ayad et al. 2007). The essential role played by P4 in maintenance of pregnancy is unequivocal and is attributed to the corpus luteum (Thatcher et al. 1986). However, the effects of P4 on the secretory patterns of placenta and its functions are not well defined. Nonetheless, the lower production of P4 by the corpus luteum may have contributed to early NT pregnancy lost. In pregnant cows, P4 can regulate genes that are responsible for availability of energy sources for embryo and up-regulate them during implantation advancing conceptus development (Forde et al. 2009). The supportive role of P4 through the endometrial environment may promote adequate conceptus elongation (Clemente et al. 2009). In addition to changes in the endometrium, P4 may have direct effect on embryo, as P4 receptor mRNA has been found in embryos (Clemente et al. 2009). In this study, as all recipients from both groups were

chosen and kept under same conditions, the considerably lower P4 concentration in NT recipients at day 80 could not be explained by effects of body condition or lactation (Santos et al. 2001). Comparison of aborted NT gestations at all stages also revealed that the P4 concentration was not significantly lower than the continuing NT pregnancies and control pregnancies at Day 80 (Fig. 10) even though it was numerically lower.

In the present study, PSPB concentrations varied from 3.61 ng/ml to 65.09 ng/ml between day 80- to 240 in the control group. Similar results have been reported in high-producing dairy cows where day of gestation was a source of variation, were observed in peripheral PAG1 concentration (Serrano et al. 2009), another isoform of the same protein (Zoli et al. 1991) . The results showed that main source of variation in PSPB concentration from controls is the aborted NT gestations (Fig. 10) especially at day 150. This timeframe may be critical for NT recipients, as the highest abortion rate at the interface of the first and second thirds of gestation (Kohan-Ghadr et al. 2008). The comparison of the NT recipients who gave birth to normal offspring versus controls reveals slight deviation that follows the same pattern of changes throughout the study period. This agrees with the result from previous reports where the investigators showed the other member of pregnancy associated proteins, PSP60 measurement as an possible predictor of anomalies in NT recipients (Heyman et al. 2002). We report also the similar pattern of changes in P4 and PSPB level in pathological nuclear transfer pregnancies.

Pregnancy specific protein in cattle is produced by the trophoblastic cells of the fetus and its secretion is specific to pregnancy, and serves as a reliable indicator of viable

pregnancy beginning the fourth week post-service (Szenci et al. 1998). While the specific function of pregnancy associated proteins (PSPB, PAG and PSP60) remains unknown, plasma concentrations of this proteins in cows are correlated with the stage of gestation. A potential effect of genetic distance between fetuses and dams on PAG concentration has been reported (Zoli et al. 1992), but in the present study, no difference was observed even though different bulls have been used. Furthermore, Lopez-Gatius et al. (Lopez-Gatius et al. 2007) reported that milk production and number of fetuses were correlated to PAG concentrations. In the present study, multiple pregnancy did not represent a factor of variation, since all pregnancies were single. Recent study was also shown that P4 concentration in maternal blood during first trimester of gestation in cows is positively correlated with PAG level reference. High P4 concentration in recipient circulation is believed to be related to abnormal prolongation of gestation in NT pregnancies (Shah et al. 2007) which was not observed in the present study.

Conclusion

The study contributes to new information on hormonal profile on somatic nuclear transfer-derived pregnancies in dairy cattle. Changes in the hormonal profile and, more specifically, the increase in E2 concentration supports the clinical observations and could be potentially used as a diagnostic tool for impaired pregnancies. As previously reported, Day 150-180 of pregnancy seems to represent a critical time in the NT pregnancy maintenance. Based on clinical observations and hormonal profile, we conclude that the

critical time when the fetal or mother life is threatened (between Days 150 and 180) can be predicted.

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Tables and Figures

			Slope		
	Group	Intercept	Estimate	Std. Err.	P. Value
Progesterone (P4)	Control	5.5504	-0.3446	0.1904	0.0779
	NT	2.6059	0.1633	0.08015	0.0453
Estrone Sulphate (E1S)	Control	0.1173	1.1275	0.4069	0.0084
	NT	-0.5332	0.8313	0.1983	<.0001
Estradiol 17-β (E2B)	Control	12.4209	13.085	3.2347	0.0002
	NT	65.8223	5.3721	1.7234	0.0026
PSPB	Control	-23.4125	17.458	2.5508	<.0001
	NT	-16.3398	14.9893	2.2282	<.0001

Table II - Relationship between hormones concentration in maternal circulation and placentome size.

The model indicated positive and significant relationships ($P<0.05$) between placentomes size and hormones (E1S, E2B and bPSPB) in both groups of pregnancy. The only exception is P4 level in Control group which was negative but non-significant.

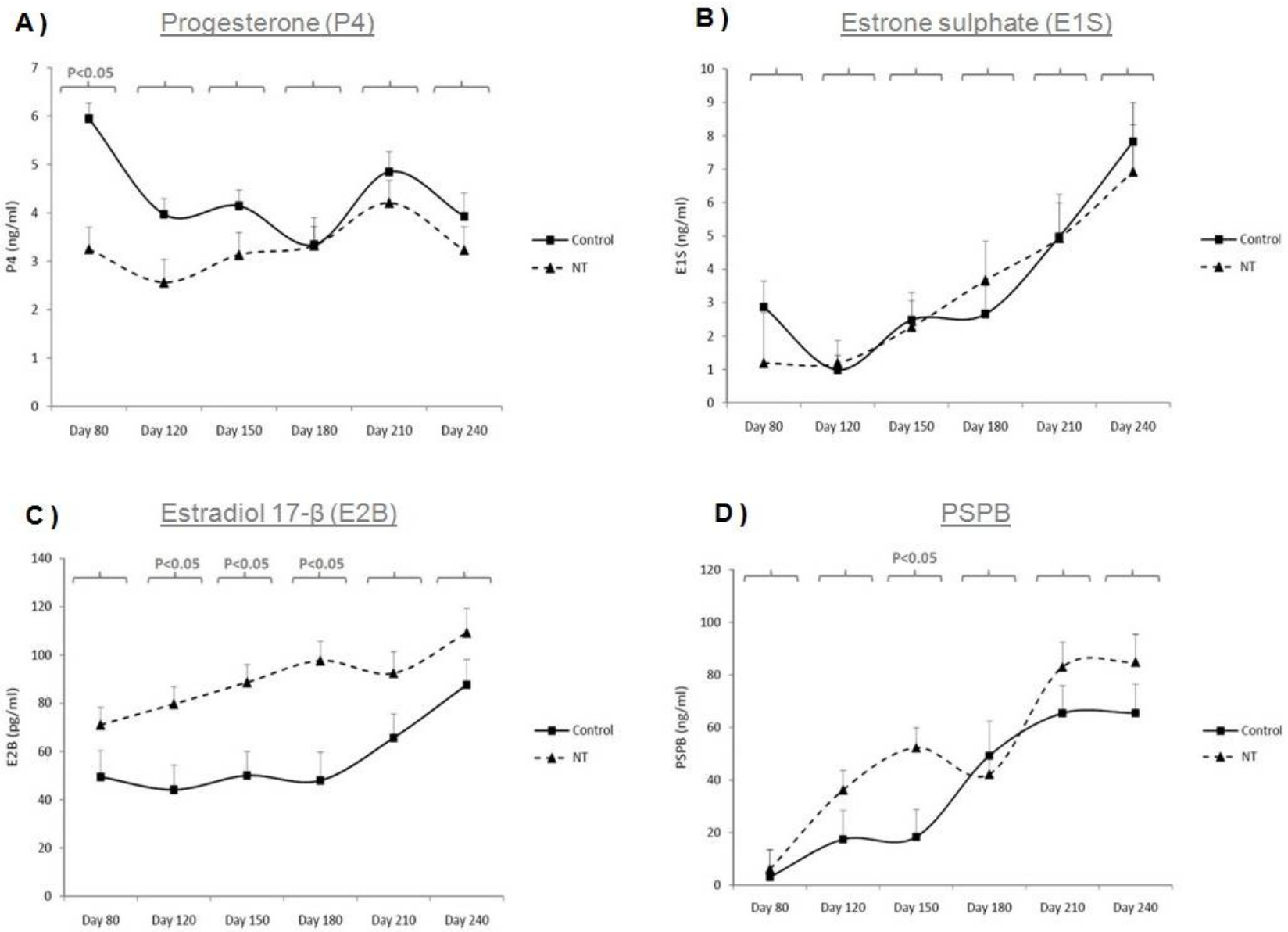
A)
Progesterone (P4)
P<0.05
P4 (ng/ml)
Day 80
Day 120
Day 150
Day 180
Day 210
Day 240
Control
NT
B)
Estrone sulphate (E1S)
E1S (ng/ml)
C)
Estradiol 17-β (E2B)
P<0.05
P<0.05
P<0.05
E2B (pg/ml)
D)
PSPB
P<0.05
PSPB (ng/ml)

Figure 9 - Changes in the hormones progesterone (P4), Estrone sulphate (E1S), Estradiol-17β (E2) and bovine pregnancy specific protein-B (PSPB) during second and final thirds of gestation in NT and control recipients (LSM±SEM).

A) Significantly lower P4 concentration in NT cows at Day 80 tends to change toward the end of gestation as it reaches approximately the same level in Controls at 3rd trimester. B) Although a steady increase in E1S occurred in both groups throughout the study period, no significant difference between groups was observed. C) At all stages of gestation the concentration of E2 in the NT recipients peripheral circulation remained higher than controls when at certain times the difference were significant as indicated by $P<0.05$. D) The level of PSPB increased from Day 80 in both groups and a difference between two groups was evident at Day120 when the PSPB was higher in nuclear transfer pregnancies. $P<0.05$).

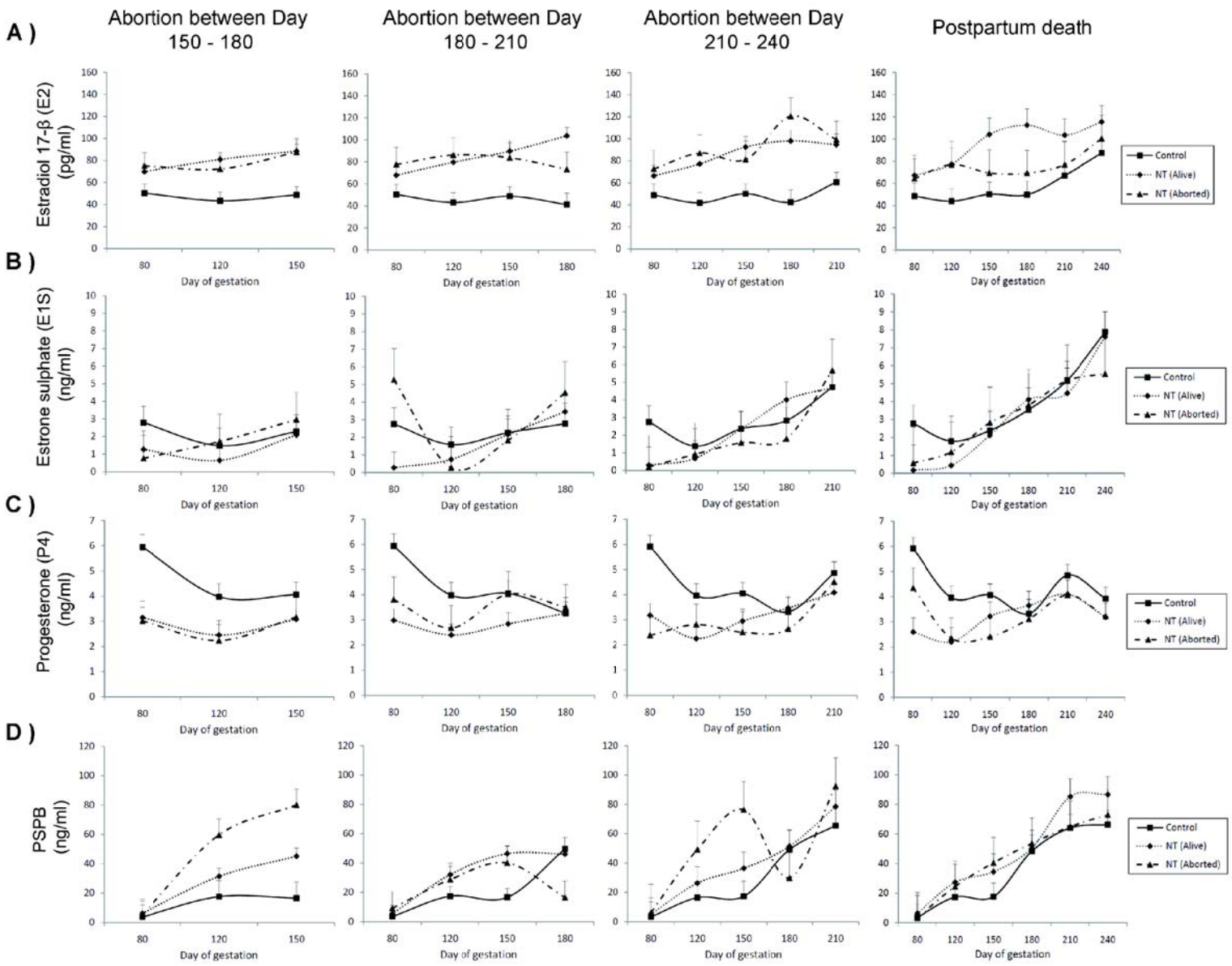
A)
Abortion between Day 150 - 180
Abortion between Day 180 - 210
Abortion between Day 210 - 240
Postpartum death
Estradiol 17-β (E2) (pg/ml)
B)
Estrone sulphate (E1S) (ng/ml)
C)
Progesterone (P4) (ng/ml)
D)
PSPB (ng/ml)
Day of gestation
Control
NT (Alive)
NT (Aborted)

Figure 10 - Comparison of hormone concentrations in two groups of alive NT and aborted NT vs. controls categorized by different stages when the failure occurred.

A) Although E2 concentration in both alive and aborted NT gestations at all stages was higher than controls, the deviations of aborted NT pregnancies are more obvious for only two time periods. For those that lost pregnancy between D180 and D210, the peripheral E2 level was suddenly dropped down about 30% at D180. Also, neonatal mortality occurred in cows with lower E2 serum concentration at earlier stages. B) E1S profile between alive and aborted NT gestations was different for recipients that lost their fetuses between Day 180 to 210 and Day 210 to 240. Interestingly, in both groups an increase in E1S concentration coincided with a decrease in E2 level. C) Comparison of aborted NT pregnancies at all stage also revealed that the P4 concentration was not considerably different from the continuing NT gestations and control pregnancies. D) The difference in peripheral PSPB concentration between aborted and non-aborted NT recipients was significant for lost pregnancies between D150 and D180. Also, in next stage aborted NT recipients had lower PSPB concentration. This deviation from the other non-aborted NT recipients can be a sign of placental dysfunction and could be used as an early clinical sign of fetal mortality at this stage of gestation.

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ARTICLE IV: Aberrant Expression of E-Cadherin and β-Catenin Proteins in Placenta of Bovine Embryos Derived from Somatic Cell Nuclear Transfer

Status: In preparation

Aberrant Expression of E-Cadherin and β-Catenin Proteins in Placenta of Bovine Embryos Derived from Somatic Cell Nuclear Transfer

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Abstract

Altered placental development appears to be a common abnormality in bovine somatic nuclear transfer (SCNT)-derived embryos. Little is known of molecular mechanisms that regulate the bovine trophoblast development during early placentation. In the present study we characterized the expression of E-cadherin and β-catenin, structural proteins of adherens junctions, in SCNT gestations as a model for impaired placentation. Cotyledonary tissues were separated from pregnant uteri of SCNT gestations (N=6) and control pregnancies (N=8) obtained by artificial insemination (AI). Samples were analysed by Western blot, quantitative RT-PCR and immonohistochemical analysis. The bovine trophectoderm cell lines from the groups (SCNT and control) were also analysed in parallel as a control for in utero condition. Although no significant differences in E-cadherin or β-catenin mRNA abundance were observed in fetal tissues between the two groups, proteins encoded by these genes were significantly ($P < 0.05$) under-expressed in SCNT trophoblast cells. Immunohistochemistry revealed a different pattern of E-cadherin and total β-catenin localization in SCNT placentas compared to the control group. No difference was observed in sub cellular localization of dephosphorylated active-β-catenin protein in SCNT tissues compared to that of control group. However, qRT-PCR confirmed that the WNT/β-catenin signalling pathway target genes *CCND1, CLDN1* and *MSX1* were significantly down-regulated in SCNT placentas. No differences were detected at any level between two groups of BT cell lines. Our results suggest that impaired down-regulation of E-cadherin and β-catenin protein expression, along with defective β-catenin signalling activation

during embryo attachment, specifically in the window of placentation, is a molecular mechanism to explain insufficient placentation in bovine SCNT-derived embryos.

Introduction

Nuclear transfer using somatic cells was first successfully achieved in sheep (Wilmut et al. 1997). Since then, the technique has been established in various species (Baguisi et al. 1999; Wakayama et al. 1999; Lee et al. 2005; Gomez et al. 2006). Although somatic cell nuclear transfer (SCNT) has good potential for applications such as therapeutic cloning, its efficiency still remains low (Hashizume et al. 2002; Heyman et al. 2002). In cattle, the success of NT gestations from the reconstructed zygote to term was reported to be as low as 5% (Hashizume, Ishiwata et al. 2002; Campbell et al. 2005). However, a study recently reported success rate of 40% in cloned pregnancies (Kohan-Ghadr et al. 2008). The abnormal placental development observed in cloned embryos and fetuses could explain early and late gestational failure (Hill et al. 2000; Heyman, Chavatte-Palmer et al. 2002; Kohan-Ghadr, Lefebvre et al. 2008). Variable clinical anomalies related to inadequate placentation have been reported in SCNT-derived pregnancies, including large offspring syndrome, hydroallantois, increased placental weight, edema, and placentomegaly (Hill et al. 2001; Constant et al. 2006; Kohan-Ghadr, Lefebvre et al. 2008). Histological abnormalities, such as degenerated and necrotic cotyledons, allantoic hypoplasia or disappearance of epithelial layer have also been described in cloned placenta (Hill, Burghardt et al. 2000; Kohan-Ghadr, Lefebvre et al. 2008).

Placenta formation and differentiation are complex procedures that rely on a series of interactions between the trophoblast and endometrium. Adhesion molecules play an important role in these events (Burghardt et al. 2002; Spencer et al. 2004; Pafilis et al.

2007). The structural and signalling molecule, β-catenin, a member of the armadillo family of proteins, is an important component in epithelial cells that functions in two different ways based on its localization. The presence of β-catenin at the plasma membrane is necessary for cell architecture as it forms a complex with α-catenin to anchor E-cadherin to actin cytoskeleton (Kemler 1993). It also regulates vital cell events such as proliferation, migration and differentiation by acting as a transcriptional regulator for several genes in the nucleus when the canonical WNT pathway is triggered (Goldstein et al. 2006; Li et al. 2006). Essential roles of canonical and non-canonical WNT pathways have been demonstrated by in vivo approaches. Addition of WNT3a to *CDX2* knockdown mouse embryonic stem cells stimulated *CDX2* expression and induced formation of trophoblast stem cells (He et al. 2008). In another study, WNT7b mutant mice died at mid-gestation stage because of placental abnormalities (Parr et al. 2001). Deletion of *TCF1* and *LEF1* transcription factors that are implicated in the WNT signal resulted in embryos with defective placenta (Galceran et al. 1999). Placental vasculogenesis was disrupted when the WNT receptor gene *FZD5* was silenced in mouse embryos (Ishikawa et al. 2001). Also, nuclear accumulation of β-catenin during embryogenesis after activation of canonical WNT signalling has been shown to promote the invasive trophoblast differentiation in the human placenta that contributes to trophoblast hyperplasia (Pollheimer et al. 2006). Recently, a report revealed that lack of β-catenin in null-mutant mouse embryos causes ectodermal cell detachment (Haegel et al. 1995) and deformity in embryonic body axis formation (Huelsken et al. 2000). In mature bovine binucleate cells, cytoplasmic and nuclear redistribution of β-catenin plays an important role in cell differentiation and maturation by

regulation of its target genes such as Cyclin D1 and c-Myc (He et al. 1998; Tetsu et al. 1999; Nakano et al. 2005).

E-cadherin is an important adhesion molecule that constructs a specific type of calcium-dependent cell to cell adhesion in epithelial cells known as adherens junctions. These junctions are responsible for extra-cellular weak adhesion by creating trans-oligomers between cadherins on opposing cell surfaces (Brieher et al. 1996) that are made stronger by lateral clustering of cadherins an event that which depends on the linkage of E-cadherin/β-catenin complex to actin-binding protein α-catenin (Aberle et al. 1994; Huber et al. 2001). The literature suggests that E-cadherin is essential during embryogenesis and placenta formation, as E-cadherin is believed to be under-expressed in mesodermal cells during gastrulation in mouse embryos (Butz et al. 1995). Also, E-cadherin knockdown mice embryos failed to form trophectoderm epithelium or a blastocyst cavity at the blastocyst stage (Larue et al. 1994). Sub-cellular localization of E-cadherin in pre-attachment bovine embryos reveals its distribution changes from apolar in the inner cell mass to basolateral in trophectoderm cells (Barcroft et al. 1998). Impaired E-cadherin expression was reported in several studies on human gestational diseases (Li et al. 2003; Xue et al. 2003; Yurdakan et al. 2008).

Previously, we reported local disappearance of placental epithelial cells, an anomaly that contributes to abnormal cotyledonary detachment in SCNT placentomes collected from advanced gestation (Kohan-Ghadr, Lefebvre et al. 2008). The goal of the current study is to investigate levels of the E-cadherin and β-catenin transcripts and protein in the

cotyledonary tissue from SCNT-derived embryos in aid of determining whether biological malfunction in the adherens junction proteins is associated with placental anomalies and insufficiencies that threaten the continuation of pregnancy.

Materials and Methods

Animals, Embryo Production and in vivo tissue collection

All animal treatment protocols were approved by the Comité de déontologie, Faculté de médecine vétérinaire, Université de Montréal in accordance with regulations of the Canadian Council of Animal Care. Animals and embryo production protocols have been described previously (Arnold et al. 2006; Vajta et al. 2006; Kohan-Ghadr, Lefebvre et al. 2008) , as has the SCNT embryo production procedure (Arnold, Lefebvre et al. 2006; Vajta, Lewis et al. 2006). Control pregnancies were derived from artificial insemination (AI) of Holstein heifers with Holstein bull's semen. At day 40, the recipients bearing viable fetuses were confirmed via ultrasonography and slaughtered (AI, N=8; SCNT, N=6). The uteri were collected at the abattoir and transported on ice to the laboratory. Cotyledonary tissues from the mid-region of the uterine horn near the fetus were collected. The collected tissues were stabilized in RNA-later (Qiagen, Mississauga, ON, Canada) and stored at -80° C for RNA extraction. To obtain protein extracts, cotyledonary tissues were snap-frozen and stored at –80 °C until further processed. The sections from placentomes were fixed in paraformaldehyde (PAF) 4% w/v overnight and kept at 4 °C in phosphate buffered saline (PBS) 1X until paraffin embedding for immunohistochemical analysis.

Cell Culture

Bovine trophectoderm cell lines from two groups of embryos (AI, N=4 and SCNT, N=4) were provided by Dr. N.C. Talbot (USDA, ARS, ANRI, Biotechnology and Germplasm Laboratory, Beltsville, MD , USA.)(Talbot et al. 2007). The cells were grown and maintained in Dulbecco's modified Eagles medium with low glucose/M199 (GIBCO, Invitrogen, USA) supplemented with 10% FBS (GIBCO, Invitrogen, USA), 0.1 nM 2-Mercaptoethanol(Sigma-Aldrich, USA), 2.5mM N-Acetyl-L-cysteine (Sigma-Aldrich, USA), 50 U/ml penicillin and 50 µg/ml streptomycin (Pen-Strep, GIBCO, Invitrogen, USA), in a humidified atmosphere of 5% CO2 at 37 °C. The medium was changed every two days. When cells reached confluence, they were scraped and gently titurated with a syringe to cell clumps. The clumps were then transferred into a new gelatin-coated petri dish to passage the line.

RNA isolation and cDNA synthesis

Total RNA was isolated either from tissues or cells using the RNeasy Mini Kit (Qiagen Mississauga, ON, Canada), according to the manufacturer's instruction. Total RNA was quantified by measuring the absorbance at 260 nm using a NanoDrop Spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, Delaware). An aliquot of 1 µg of total RNA from each sample was treated with DNase kit (DNase I,

Ambion, USA) and reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen) as recommended by the manufacturers.

Real-time PCR

Quantitative real-time PCR was performed using 7300 Real-Time PCR System (PE Applied Biosystems) and SYBR green PCR master mix (Applied Biosystems). Gene-specific primers were designed based on bovine gene sequences from GenBank (Table III) and using Applied Biosystems' Primer Express 3.0 software. The specificity of amplified products was verified by dissociation curve analysis for each primer set. To evaluate PCR efficiency, standard curves were prepared for the studied genes using a pool of cDNA from all samples. PCR efficiency for each gene was co-evaluated using LinRegPCR software(Ruijter et al. 2009). Each PCR reaction comprised 10 µl of Power SYBR Green PCR Master Mix (PE Applied Biosystems), 2 µl of forward and reverse primer mixture at optimized concentrations of 300 nM, and 8 µl of cDNA template and sterile water containing 15 ng cDNA to a final volume of 20 µl. The thermal cycling conditions included one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative quantification method ($2^{-\Delta\Delta Ct}$) was used to determine the mRNA abundance of target gene (Pfaffl 2001; Pfaffl et al. 2002) and the final values were normalized against the geometric mean of three housekeeping genes (GAPDH, Cyclophilin A(PPIA) (Bettegowda et al. 2006) and H2AFZ (Portela et al.)) for each sample.

Preparation of protein lysates

For in vivo experiment, the frozen cotyledonary tissues were ground by mortar and pestle and homogenized in sonication buffer (20 mM Tris, 50 mM EDTA, 0.1 mM DEDTC, pH8.0; Invitrogen Life Technologies Corporation, Burlington, ON, Canada and Sigma-Aldrich, Oakville, ON, Canada) containing Mini Complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, GE). Supernatants were centrifuged for 10 minute at 12,000 rpm. Monolayer trophectoderm cells in plates or dishes were lysed in M-PER® Mammalian protein extraction reagent (Thermo Scientific, Rockford, USA) supplemented with Mini Complete EDTA-free protease inhibitor cocktail and phosphatase inhibitor cocktail (PhosSTOP®, Roche). The cell lysate was collected and clarified by centrifugation at 12,000 rpm for 10 min. The protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). All lysates were frozen and kept at −80°C until use.

Immunoblot Analysis

The primary antibodies employed were: E-cadherin (1:1000, Cell Signaling, #4065), total β-catenin (1:40000, Epitomics, #1274-1), active-β-catenin (1:1000, Millipore, #05-665). β-actin (1:45000, Santa-Cruz, SC-47778 HRP-conjugate) was used as loading control. In this experiment MCF7 whole cell lysate was used as positive control. Equal amounts of total protein from each lysate (tissue or cells) were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electroblotted onto PVDF membrane (GE Healthcare, Piscataway, NJ). Molecular weight markers were purchased

from Amersham Biosciences, UK and used as reference. Nonspecific antibody binding was blocked by incubation of membranes in 5 % non-fat dry milk in 0.01% Tween-20 in TBS (TBST) for 1 h at room temperature and then incubated with the primary antibody in 5% milk in TBST for overnight. After 3 washes for 5 min each, blots were incubated for 1 hour with appropriate HRP-conjugated secondary antibody (1:100,000, Jackson Immuno Research Laboratories INC, Ontario, Canada), followed by washing (3 x 5 min). Subsequently, Immobilon Western Chemioluminescent HRP Substrate (Millipore, Billerica, MA, USA) was used to detect the targeted protein. The optical density was analyzed using ImageJ v.1.31 software (National Institutes of Health, Wayne Rasband, USA).

Immunohistochemistry

To localize the target proteins histochemically, the cross-sections of paraffin embedded tissues were mounted on to histological slides. Wax was removed from sections using xylene, following by rehydration procedure through serial dilutions of ethanol and a finalrinse in distilled water. The tissue specific endogenous peroxidise activity was then inhibited by incubating slides in 0.3% hydrogen peroxide in PBS containing 0.01% sodium azide. Antigen retrieval was carried out subsequently by boiling the slides in 0.01 M citrate buffer, pH 6.0, at 97° C for 10 min. Sections were blocked with 5% bovine serum albumin (BSA) in PBS for 2 hours in a humid chamber at room temprature. After blocking of nonspecific endogenous biotin by Avidin/Biotin Blocking Kit (Vector, Burlingame, CA),

the tissues were incubated overnight at 4°C with the same primary antibodies as used in western blot procedure, against either E-cadherin (1:100) or β-catenin (1:200) or dephosphorylated active-β-catenin (1:100) in humid chamber. Corresponding nonspecific IgGs were used as negative control and processed in parallel. After 3 washes in PBS (each for 5 min), sections were incubated with a biotinylated second antibody diluted 1:200 in PBS for 1 h in humid chamber at room temperature. The following three washes in PBS were followed by incubation with Vectastain Elite® ABC kit (Vector, Burlingame, CA) for 45 min in humid chamber at room temperature. The sections were treated with 3,3'-diaminobenzidine (DAB) to detect peroxidase activity. Counterstaining was performed by 15 sec incubation in hamatoxylin stain solution (Fisher scientific, Fair Lawn, New Jersey, USA). After rinsing with tap water for 10 min, the sections were dehydrated, cleared in xylene and a coverslip was added with histological mounting medium (Permount®, Fisher scientific,).

Immunocytochemistry in trophoblast cell culture

Bovine trophoblast cells cultured on collagen-coated coverslips were washed twice in PBS (5 min each) and then fixed with 4% paraformaldehyde (PAF) in PBS (pH=7.4) at room temperature for 20 min. After 4 washes with PBS, the cells were permeabilized by a wash with PBS containing 0.2% Triton X-100 followed by four washes with 0.05% Tween-20 in PBS (5 min each). The cells then were blocked in 5% bovine serum albumin (BSA) in PBS for 30 min. Incubation with primary antibody (diluted 1:100) was carried out in 5% BSA/PBS supplemented with 0.5% Tween-20 for 1 h at room temperature. After four

washes with PBS, the appropriate Cy3-conjugated second antibody in 5% BSA/PBS was applied for 1 h at room temperature. The next two washes with 0.05% Tween-20/PBS was followed by nuclear staining by DAPI diluted 1:1000 in PBS for 5 min. After one wash with PBS, the cells were mounted on histological slides using Perma Fluor, and viewed under an epifluorescence microscope.

Statistical Analysis

Statistical analysis was carried out using JMP, Version 7 (SAS Institute Inc., Cary, NC, USA) and STATISTICA 8.0 (StatSfoft, Inc., Tulsa, OK, USA). Non-parametric analysis was applied (Wilcoxon/Kruskal-Wallis test, Rank Sums) to assess the significance of differences. Two sample means were compared by Wilcoxon rank sum test. Gene data are presented as mean ±SEM and statistical difference was recognized when $P < 0.05$.

Results

Expression analysis and immunolocalization of E-cadherin and β-catenin proteins

The western blot analysis using anti total β-catenin and anti E-cadherin antibodies detected specific signals at about 94 kDa and 135 kDa respectively in both placental tissue extracts and cell line lysates. Expression of both β-catenin and E-cadherin was significantly lower in SCNT cotyledonary tissues compared with those of the control group. However, no significant differences were seen between two groups of bovine trophoblast cell lines for

either total β-catenin (Fig.13A,13B) or E-cadherin (Fig.13A,13C). E-cadherin protein was localized by immonuhistochemistry to control and SCNT placentomes. In control tissue, E-cadherin was detectable in both cytoplasm and membranes of placental epithelium. However, the expression of E-cadherin was more associated with the membrane than with the cytoplasm in mononucleate placental cells while the binucleate cells of trophoblast provenance displayed stronger cytoplasmic expression (Fig. 11A). The E-cadherin protein signal was significantly lower in SCNT placenta compared to controls (Fig. 11B). The pattern of distribution was also different. In SCNT, the staining was limited to cytoplasm and no clear membrane signal was observed. Membrane immunostaining of total β-catenin protein was observed in control placental epithelium in both mononucleate and binucleate cells. The cytoplasmic presence of β-catenin was also detectable in the latter (Fig. 11D). Unlike the control, pale membrane staining of total β-catenin was detected in both cell types of SCNT placenta. Also, the cytoplasmic expression of total β-catenin appeared lower in binucleate cells of SCNT placenta (Fig. 11E). No obvious difference was observed by immunofluorescent localization of E-cadherin (Fig.12 G-L) and β-catenin (Fig.12 A-F) between control and SCNT trophoblast cell lines. Faint membrane localization of dephosphorylated active β-catenin with dispersed cytoplasmic staining was noted in both control and SCNT placenta, with no apparent difference (Fig. 11F,11G).

Analysis of E-cadherin (CDH1) and β-catenin (CTNNB1) mRNA expression

Quantitative RT-PCR analysis demonstrated that no significant difference in either β-catenin (*CTNNB1*) or E-cadherin (*CDH1*) mRNA abundance level was detected between control and SCNT Day 40 placental tissues. Similar results were observed in comparison of control and SCNT trophectoderm cell lines (Fig. 14).

Expression profile of WNT/β-catenin signalling target genes

Quantitative real-time RT-PCR analysis of 4 active-β-catenin regulated downstream genes (*CCND1, TP53, CLDN1 and MSX1*) showed that at least three out of four (*CCND1, CLDN1 and MSX1*) were down-regulated in SCNT Day 40 cotyledonary tissues compared to that of control. However, no significant differences were detected between control and SCNT cell lines (Fig. 14).

Discussion

The present study demonstrated reduced expression of E-cadherin and total β-catenin protein trophectoderm epithelial cells during the window of placentation in SCNT derived embryos. The results further identified an abnormality in localization pattern of both proteins at sub-cellular level in Day 40 SCNT placenta compared to that of normal gestations.

In the bovine placenta, the importance of β-catenin and E-cadherin in differentiation of trophoblast binucleate cells is emphasized by the observation of the dynamic rearrangement in adherens junction mediated cell adhesions during cell migration (Nakano, Shimada et al. 2005). The basolateral membrane localization of E-cadherin and β-catenin in trophectoderm cells of pre-attachment bovine embryos underlines their role in maintaining trophoblast stability even at early stages before BNCs formation (Barcroft, Hay-Schmidt et al. 1998). Our previous study reported loss of placental attachment in SCNT placentomes in advanced gestation and a disappearance of trophoblast epithelial cells that might cause the placental insufficiency and failure of pregnancy (Kohan-Ghadr, Lefebvre et al. 2008). In the present study, we show that two main constructive components of apical adhesion complex, β-catenin and E-cadherin proteins were under-expressed in the Day 40 SCNT bovine placenta. Lower level of E-cadherin expression was previously reported in SCNT bovine embryos reconstructed with fetal fibroblasts (Jang et al. 2005).The investigation also confirmed that the membrane distribution of both proteins was reduced in SCNT trophoblast cells. These observations suggest that aberrant down-regulated E-cadherin and β-catenin protein in trophoblast cells plasma membrane of SCNT bovine placenta during early placentation could weaken the structure and integrity of trophoblast cells and result in loss of attachment. This, in turn could compromise the nutrient support of the embryo and eventually, results in pregnancy failure. A similar situation was observed in the human placenta, where impaired differentiation in cytotrophoblasts resulted in spontaneous abortion (Yurdakan, Ekem et al. 2008). An immunohistochemical study revealed that in human gestational trophoblastic diseases including hydatidiform moles, choriocarcinoma,

and PSTT (placental site trophoblastic tumour), both E-cadherin and β-catenin were down-regulated in pathological placentas (Li, Cheung et al. 2003).

The present study demonstrated membrane and cytoplasmic localization of dephosphorylated β-catenin in trophoblast cells in both control and SCNT placenta. Generally, nuclear localization of active β-catenin is considered to be correlated with WNT activation. However, upon WNT stimulation, dephosphorylated β-catenin is also recruited to the plasma membrane (Hendriksen et al. 2008). Taking into account the initial results, reduced β-catenin expression in SCNT placenta could represent the alteration its transcriptional activity. The expression of several WNT/β-catenin signalling responsive genes might control trophoblast cell proliferation and differentiation. The present study measured a significant down-regulation of *MSX1* in SCNT placenta. However, no difference was observed in BT-1 transcript profile. *MSX1* is a member of homeobox transcription factors family that functions as inhibitor of tissue specific bHLH transcription factors. Different studies demonstrated that *MSX1* is the downstream targets of the WNT/ β-catenin signalling pathway (Willert et al. 2002; Stevens et al. 2003; Song et al. 2009). *MSX1* is believed to revert differentiated myotubes into an undifferentiated state (Odelberg et al. 2000). In addition, expression of *MSX1* was correlated to pre-implantation embryo death in bovine (El-Sayed et al. 2006).

The considerably less cyclin D1 (*CCND1*) mRNA expression in SCNT fetal membrane is expected to affect the trophoblast cell differentiation and BNCs formation. Recent results demonstrated that the positive correlation of the intracellular level of β-catenin with DNA content of binucleate cells implies the involvement of WNT/β-catenin

signalling in the onset of endoreduplication in binucleate cells through regulation of responsive genes like *CCND1* (Nakano, Shimada et al. 2005). Considering previous reports, the present results provide evidence to support the hypothesis that alteration in β-catenin protein level in SCNT placenta affects the size of the population of BNCs and explain the fewer numbers of BNCs in SCNT cotyledonary tissue compared to that of normal pregnancies (Arnold, Lefebvre et al. 2006).

The present study also revealed that Claudin-1 (*CLDN1*) is down-regulated in SCNT fetal tissue. Claudin-1 is identified as a membrane protein associated with tight junctions that acts as an important barrier in cell-cell contacts in epithelial and endothelial cells (Tsukita et al. 2002). *CLDN1* is identified as a target for β-catenin transcriptional activation in human cancers (Miwa et al. 2001). Also, it is positively correlated to regulation of the expression and sub-cellular localization of β-catenin and E-cadherin (Dhawan et al. 2005; Oliveira et al. 2007; Akasaka et al. 2010). *CLDN1* modulation contributes to the cellular resistance to apoptosis (Akasaka, Sato et al. 2010), cell migration, anoikis and invasion (Dhawan, Singh et al. 2005). Thus, any aberration in *CLDN1* expression has biological significances in trophoblast sub-cellular structure and population.

Together, our qRT-PCR results suggest that expression of some of the WNT/β-catenin signal target genes declines in SCNT trophoblast cells. The consequences of this decline may be compromised fetal wellbeing through perturbation in placental function.

However, further studies are required to investigate the alteration of WNT/ β-catenin downstream genes at the protein level to clarify and expand current results.

Previous studies have demonstrated that the cadherin-catenin complex functionality is controlled by the balance between kinase and phosphatase activities (Lickert et al. 2000; Bek et al. 2002; Nelson et al. 2004). Phosphorylation of serine/threonine sites were found to be necessary for E-cadherin redistribution during compaction stage in mouse embryos (Sefton et al. 1996). Also, activation of tyrosine kinases in MDCK cells resulted in a loss of cadherin-mediated cell-cell adhesion that accompanies by occurrence of cadherin endocytosis (Le et al. 1999). In the present study, although E-cadherin and β –catenin proteins were under-expressed in SCNT placenta, no alteration in *CDH1* and *CTNNB1* mRNA expressions was observed in SCNTs. In the other hand, abnormal cellular distribution of both E-cadherin and β –catenin proteins was observed by immunohistochemistry. Collectively, the present observations suggest that post-transcriptional or –translational mechanisms might regulate their expression in trophectoderm cells.

In conclusion, the results suggest that E-cadherin and β-catenin play a critical role in bovine trophectoderm formation and function. Defective activation of β-catenin during placentation (Day 40) in SCNT derived fetus could compromise the feto-maternal attachment and exchange and eventually the embryo and fetus survival resulting in pregnancy loss. However, further studies will be required to investigate the possible post-transcriptional and/or post-translational regulatory mechanisms that influence E-cadherin and β-catenin expression in trophoblast cells during SCNT gestation to provide further

insight into the underlying events that control adherence and attachment of trophectoderm in early gestation.

Acknowledgements

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Tables and Figures

Gene	Gene Bank Accession number	Primer sequence 5' to 3' Forward, Reverse	Reference
CTTNB1	NM_001076141.1	GATGAGGACCAGGTGGTGGTT, CCATCTGAGGAGAACGCATGA	
CDH1	BC147914	CCAGGAACCTCTGTGATGCA, TGGGATCTTGGGCTAGGATTT	
TP53	NM_174201	GCACATGACGGAGGTTGTGA, TCCCTTCCACCCGGATAAG	
CCND1	NM_001046273	GGCCGAGAAGCTGTGCATT, CGCCAGGTTCCACTTGAGTT	
CLDN1	NM_174201	GCACATGACGGAGGTTGTGA, TCCCTTCCACCCGGATAAG	
GAPDH	NM_001034034.1	GCCATCAATGACCCCTTCAT, TGCCGTGGGTGGAATCA	Bettegowda A. *et al.* (2006) [44]
H2AFZ	BC126824.1	GAGGAGCTGAACAAGCTGTTG, TTGTGGTGGCTCTCAGTCTTC	Portela VM. *et al.* (2009) [45]
PPIA	NM_178320.2	GCCATGGAGCGCTTTGG, CCACAGTCAGCAATGGTGATCT	Bettegowda A. *et al.* (2006) [44]

Table III - Primers used for Real Time PCR

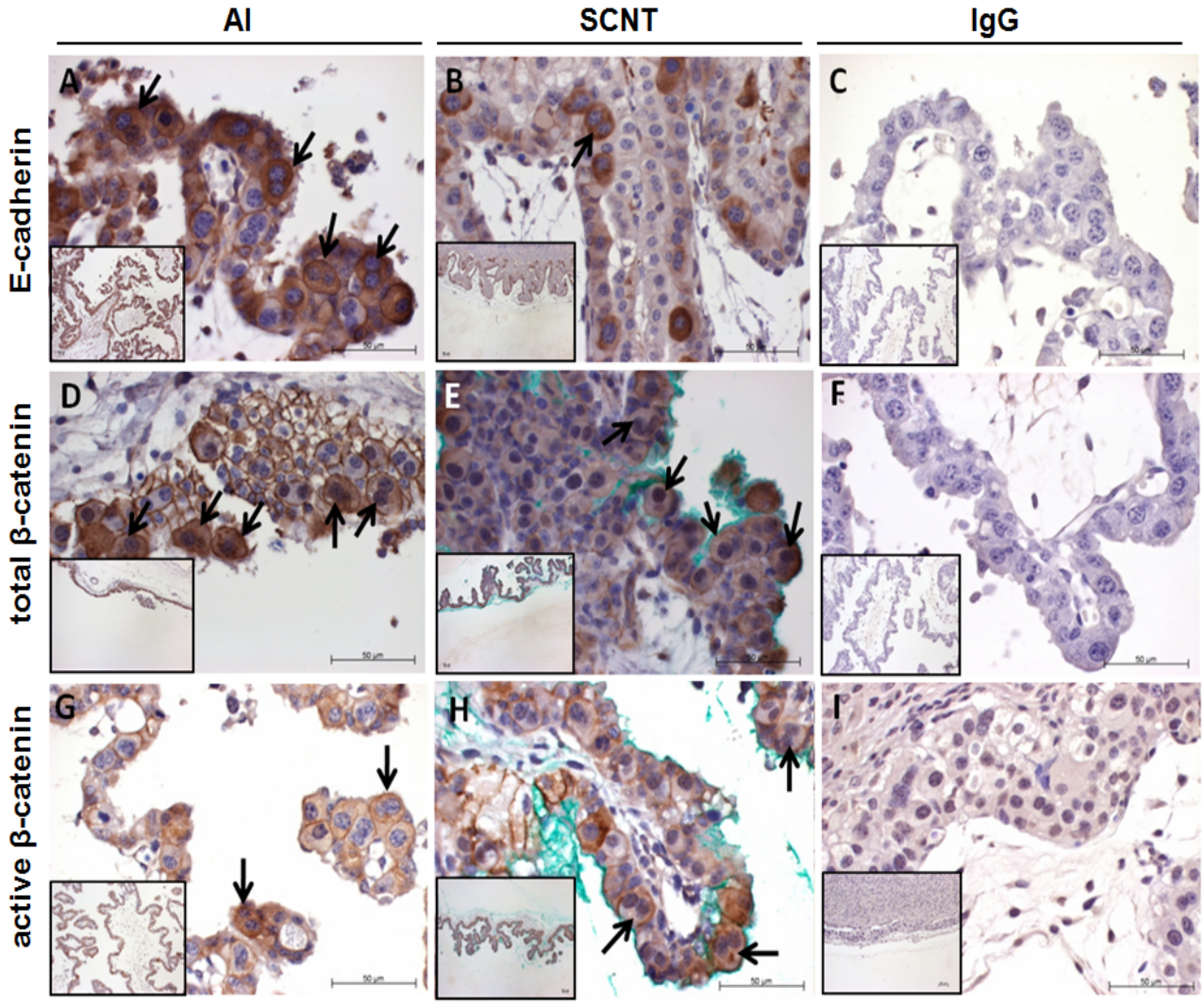
AI
SCNT
IgG
E-cadherin
total β-catenin
active β-catenin
A
B
C
D
E
F
G
H
I

Figure 11 - Immunohistochemical studies of total β-catenin, active dephosphorylated β-catenin and E-cadherin in SCNT bovine placenta.

Paraffin embedded sections of cotyledons at 40 days of gestation from AI (A,D,G) and SCNT (B,E,H) gestations were stained with a monoclonal anti E-cadherin (A, B) or monoclonal anti β-catenin (D, E) or monoclonal anti active-β-catenin (G,H) antibodies. Lower power views are also shown in each figure. IgG treated slides were used as negatives (C,F,I). Total β-catenin is more localized in cell membrane of trophoblastic cells in AI (D) compared to that of SCNT (E) placenta. E-cadherin is also disrupted in SCNT (D) compare to AI (C) as its cytoplasmic expression is very diffused (stain-like) in BNCs and no clear signal is visible at the cell boundaries. Binucleate cells (BNCs) are shown by arrows. Magnification: Main views: 630x, Lower power views: 200x; scale: 50 μm

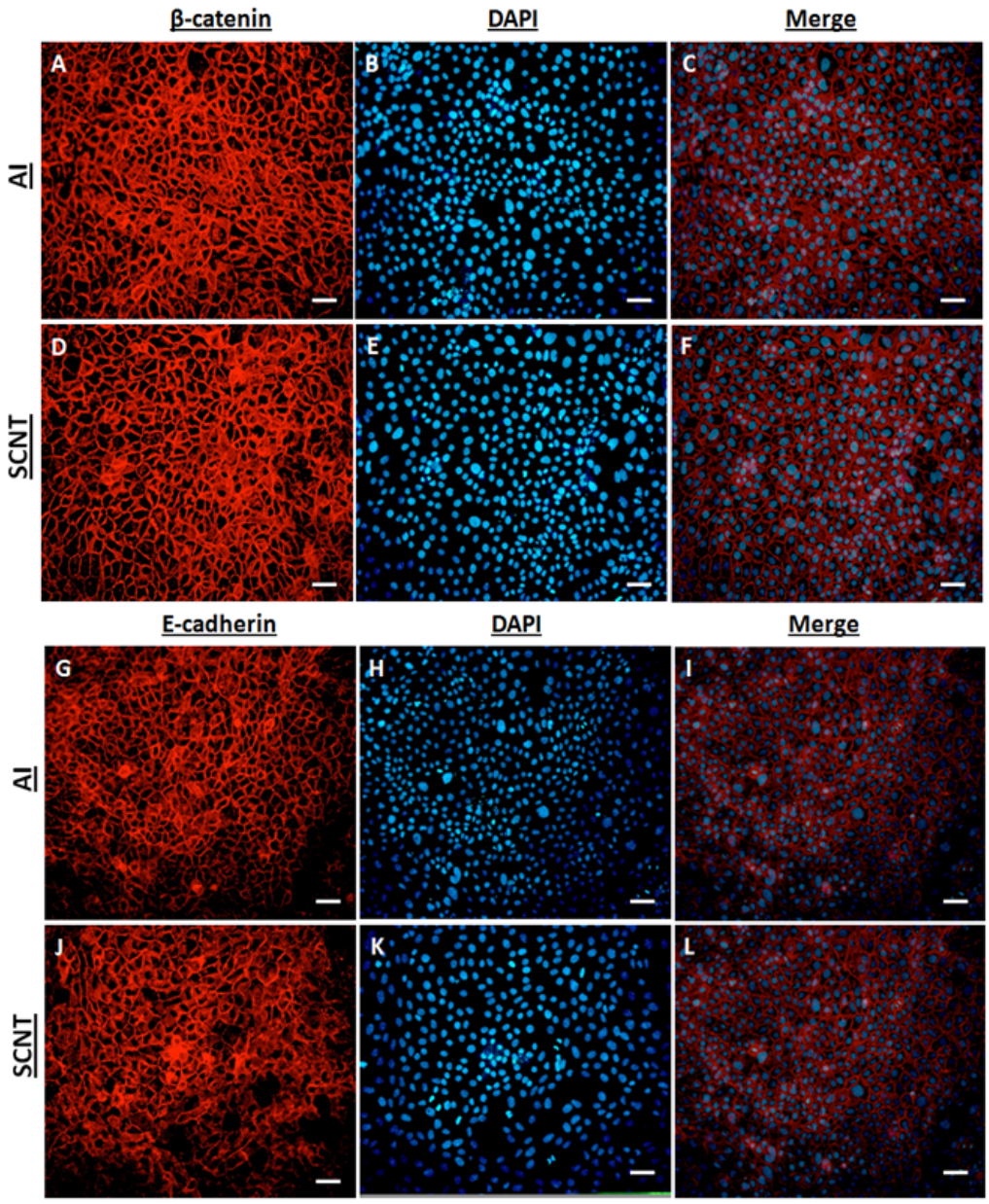

Figure 12 - Bovine Trophectoderm (BT) cells

showing similar intense membranous β-catenin in both AI (A-C) and SCNT (D-F) cell lines. E-cadherin is also stain in membranes of AI (G-I) and SCNT (J-L) cells. Magnification: 200x, scale: 50 μm.

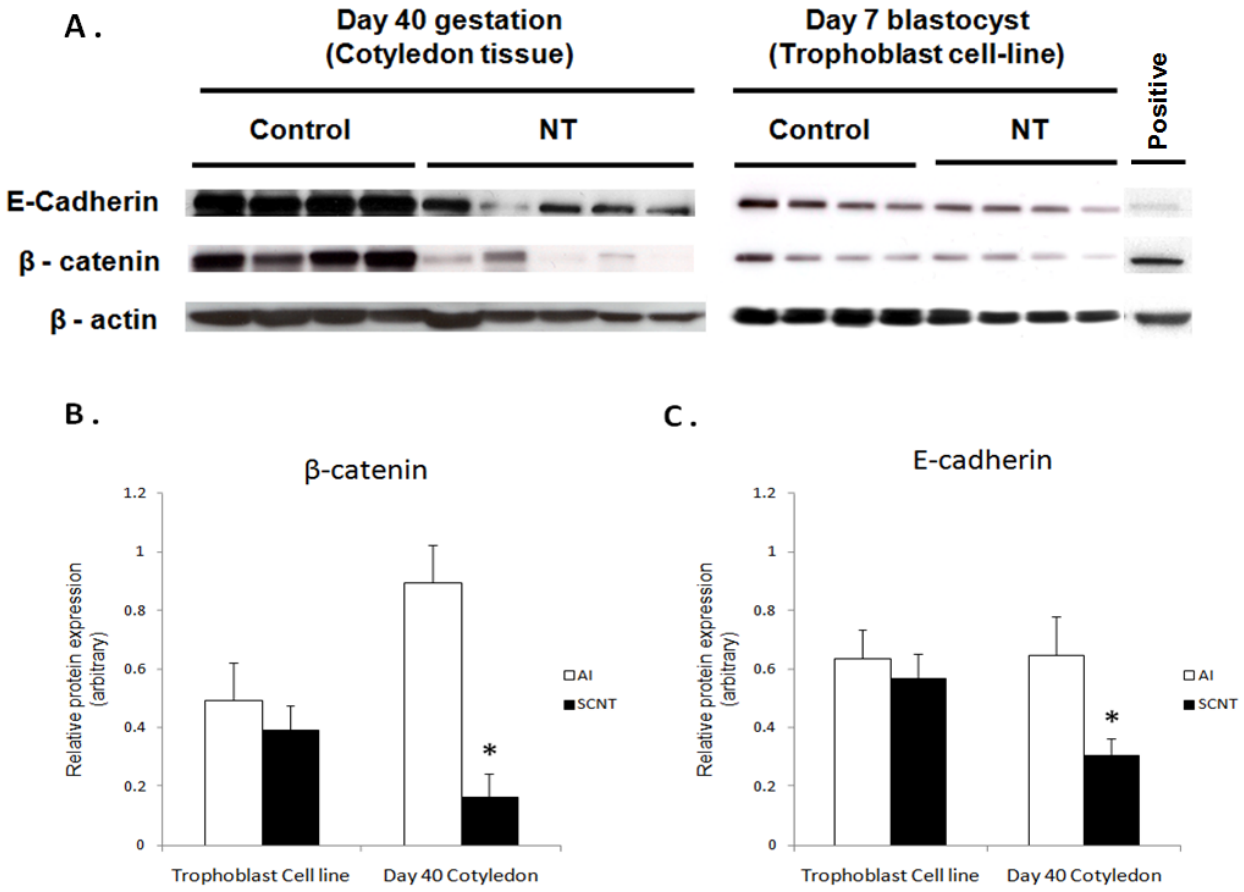

Figure 13 - Western blot analysis.

Total protein from tissue or whole-cell lysates was extracted. A.) β-catenin and E-cadherin protein were detected by Western blot. β-actin was used as a loading control. Cumulative results for quantitative densitometry of samples in each group are shown as bar graph for B.) β-catenin and C.) E-cadherin. Mean ± SEM values are depicted for protein abundance and are normalized by loading control expression. *, $P < 0.05$.

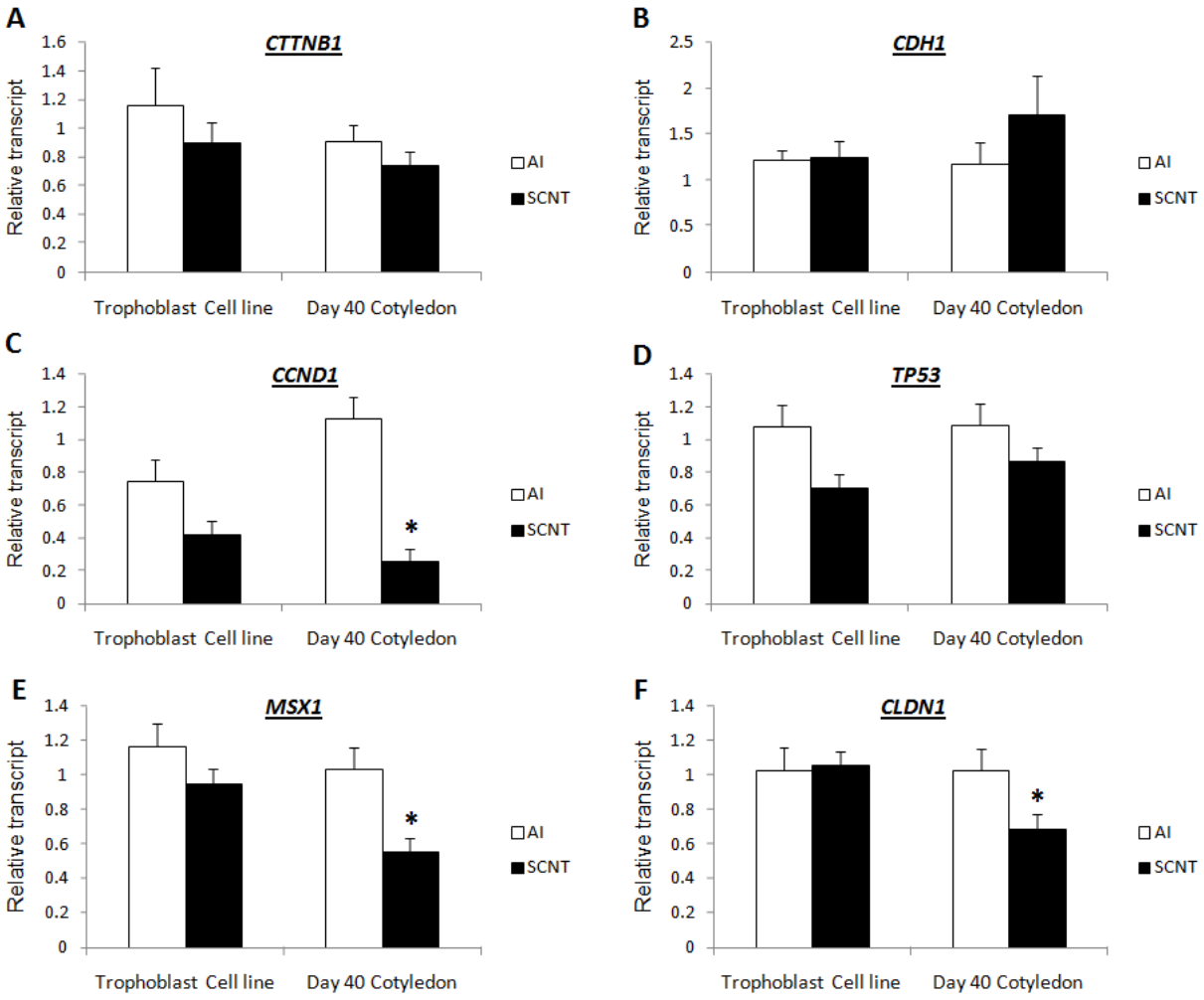

Figure 14 - Relative abundance of transcript of genes

for A) *CTNNB1* B) *CDH1* C) *CCND1* D) *TP53* E) *MSX1* and F) *CLDN1* in trophectoderm cell lines and Day 40 placenta from AI (white) and SCNT (black) gestations. Histograms represent Mean ± S.E.M of relative mRNA abundance normalized by geometric mean of housekeeping genes. *, $P < 0.05$.

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General Discussion

Abnormal placental development is a major problem associated with somatic cell nuclear transfer (SCNT). As the placenta plays an essential role in maintaining the fetus throughout gestation, pathological changes of the placenta have potentially serious consequences for embryonic and fetal development and neonatal survival. In this series of studies, we provide novel information about placental development and function throughout gestation in cattle by characterizing morphological and functional changes of placenta in traditionally produced (control) and SCNT-derived pregnancies.

The study is justified by the extensive information demonstrating that application of assisted reproductive technology compromises pregnancy in cattle. In one investigation, more than 80% of the abortions in SCNT gestations occurred between Days 30 and 60, and these losses are believed to be associated with poor placental development (Hill et al. 2000). Wells et al. (Wells et al. 1999) reported that only 10% of SCNT-produced embryos reach term and survival rates to Days 60, 100, 180 of age are 45%, 21% and 17% respectively. These authors noted that the failure of several pregnancies during the third trimester were the result of excessive accumulation of allantoic fluid, further pointing toward abnormal placental function as a potential cause of loss. This condition which, also referred to as hydrops, is rare in pregnant cattle but has been observed in several studies of SCNT cloned pregnancies (Hill et al. 1999; Wells et al. 1999; Hill et al. 2000; Kato et al. 2000; Everts et al. 2008). Other placental anomalies, such as enlarged umbilical cord, placentomegaly, abnormal placental vascularisation and placental edema, have been

frequently reported as causes of compromised cloned gestations (Cibelli et al. 1998; DeSouza et al. 2001; Chavatte-Palmer et al. 2002; Constant et al. 2006). The placental complications in SCNT pregnancies are not only life-threatening for the fetus but can also endanger recipients. As an example, the recipients with hydrops are frequently distressed, anorectic, dehydrated, weak and eventually, recumbent (Drost 2007). In the current series of experiments of the present study, a variety of approaches were used to characterize placental anomalies in SCNT gestations with a purpose of developing a strategy to identify placental anomalies for early diagnosis and early medical intervention. By these means, it will be possible to improve the overall efficiency of cloning in cattle.

At the first step, we needed to characterize the morphological changes in fetal membranes of SCNT pregnancies. As most literature focused either on dysfunction at the time of implantation or abnormalities of SCNT offspring, we designed a longitudinal study to assess placental abnormalities that could be potentially harmful for maternal and fetal well-being during 2^{nd} and 3^{rd} trimester of SCNT gestations. To do so, we monitored the SCNT recipients by ultrasonography at different stages during 2^{nd} (Days 80, 120, 150, 180) and 3^{rd} (Days 210 and 240) trimesters of gestation. A mix group of recipients bearing in vitro fertilized or frozen embryos were studied and monitored as control. An array of morphological factors, such as placentomes and umbilical cord size and shape, the thickness of amniotic and allantoic walls and echodensity of fetal fluids were recorded and measured. Also, tissue samples from placentomes, amniotic and allantoic membranes were collected either from aborted recipients or at parturition. We observed that the placentomes of NT pregnancies were larger and longer than their counterparts in control pregnancies.

Our observation was accorded to previous reports (Heyman et al. 2002; Constant et al. 2006). However, our data showed that SCNT placentomes were larger at all stages and constantly continue to grow after Day 240 when the curve of placentome size in control gestations tends to be flat. We detected mini-cotyledons from Day 210 that may represent a compensatory mechanism to sustain sufficient placental exchange starting in the 3rd trimester when a smaller number or dysfunctional placentomes are present. These mini-placentomes appeared well formed and normal, except for their size. However, they were not detectable by ultrasonography before Day 210 so we were not able to determine whether they were growing or regressing during the gestation. We also evaluated placentomes histopathologically. The presence of necrotic cells was the most frequent anomaly in SCNT placentomes. However, the distribution pattern of necrotic cells changed from discrete foci at Day 150 to to more generalized at Day 240, spreading through the epithelium of both fetal membrane and caruncles. We also reported the disappearance of the fetal membranes epithelium that may lead to placental insufficiency, pregnancy failure and eventually, reduced neonatal survival. Apart from these changes in placentome size and appearance, we report a cluster of anatomical abnormalities, including amniotic plaques and edema in fetal membranes. Hydroallantois was the most common placental anomaly in this study with the occurrence rate of 45% that was consistent with previous reports (Hill et al. 1999; Heyman et al. 2002). Based on our observations, the presence of edema can be characterized by areas of alternating hypo- and hyper-echodense bands in the thickness of the utero-chorioallantoic layer and amniotic membrane. The correlation between hydroallantois and fetal renal failure was confirmed in a study on bovine in vitro produced

pregnancies (van Wagtendonk-de Leeuw AM et al. 1998). Hence, the detection of excessive allantoic fluid in early stages could severely threaten for both feta and maternal health at later stages. In the present investigation, this condition was most commonly observed between Days 150 and 180 of cloned pregnancies and may account for pregnancy loss during this period. In addition, the presence of excessive allantoic fluid could be used as an early sign for problematic gestations. As all ultrasonographic observations were substantiated by necropsy findings of aborted or delivered SCNT fetuses, ultrasonography technique could be a reliable monitoring technique for SCNT pregnancies that could be used to predict the outcome more accurately.

Based on anatomical and histological malformations in SCNT placenta and the fact that the placenta secretes several hormones and proteins (progesterone (Reimers et al. 1985), estrogens (Matamoros et al. 1994) and pregnancy associated glycoproteins (bPAGs) (Roberts et al. 1995), we theorized that placental abnormalities and malfunctions in SCNT gestations are correlated and could thus be used as a diagnostic tool for monitoring impaired pregnancies. To test this hypothesis, we designed the second set of experiments to assess the changes in concentration of four hormones (P4, E1S, E2 and PSPB) in maternal circulation and to evaluate their association with the placental morphological abnormalities. Serum concentrations of hormones in SCNT and control recipients were measured at different stages of gestation (Days 80, 120, 150, 180, 210, and 240; Day 0 = estrus). At each stage of monitoring, we divided the SCNT group dataset to two sub groups of aborted SCNT (recipients that failed to reach the next stage) and continuing SCNT (pregnancies that reached to next stage). The significant finding of this study was that there is a higher

level of E2 in NT pregnancies at all stages. Our results showed that E2 concentration progressively increased between day 80 and day 240 of gestation to the point that it became statistically significant between Day 120 to Day 180. Importantly, this period coincides with the period when we reported the high rate of hydroallantois and fetal mortality (Day 150 to 180). We already showed that placentomes of SCNT pregnancies are wider and longer than their counterparts in control pregnancies that could show an increase in number of mono- and/or binucleate trophoblastic cells. This could explain a greater synthesis of E2 by SCNT placenta. Also, higher concentrations of E2 could be a cause of hydroallantois in SCNT placenta as excessive amounts of E2 could change extracellular fluid distribution across compartments (Greenleaf 1990). Interestingly, the SCNT recipients that lost gestations between day 180 and 210 and those who gave birth to abnormal or dead offspring showed a decrease in the mean concentration of E2 at mid-gestation compared to the recipients of normal SCNT pregnancies. So, we suggest that the deviation of E2 concentration in circulation of SCNT recipients from the mean of normal SCNT pregnancies could be used as a predictor of problematic pregnancies. However, further experimentation is required to determine the mechanism(s) that might regulate the cause and/or consequence of perturbation in E2 level on both fetus and mother in SCNT pregnancies. SCNT recipients had a lower level P4 concentration levels, in comparison to controls at the beginning of second trimester of gestation. As pregnancies progressed, the P4 pattern changed and SCNT recipients approached controls and, after Day 180, both groups followed the same pattern. Comparison of SCNT sub groups revealed that aborted SCNT gestations had no impact on the abundance of P4. However, in this study we were

not able to distinguish the P4 produced by corpus luteum from that synthesized by placenta. As the comparison of SCNT sub groups revealed no significant difference in P4 concentration between aborted and continuing SCNT pregnancies, P4 does not represent a reliable monitoring factor for prediction of placental anomalies. For E1S serum concentrations, no significant difference between the SCNT and control groups was observed. However, at an early stage (Day 80), the SCNT recipients had lower concentration of E1S. This observation is in agreement with the previous report of a comparative study on E1S profiling of bovine recipients of SCNT fetus during gestation (Shah et al. 2007). By comparison between live and aborted SCNT recipients between Day 180 to Day 240, a reverse pattern for E1S and E2 levels was noticeable, in that the increase in E1S concentration coincided with a decrease in E2 level which perhaps is an indication of changing balance between active and inactive form of estrogens but further studies are needed to confirm this. In cattle, pregnancy specific protein B (PSPB) serves as a reliable indicator of viable pregnancy beginning the fourth week post-service (Szenci et al. 1998). In this study, we observed that, PSPB concentration in NT cows differed considerably during the second trimester and we showed that the source of variation was the aborted SCNT gestations, especially those who failed shortly after day 150. The comparison of the NT recipients who gave birth to normal offspring versus controls reveals slight deviation that follows the same pattern of changes throughout the study period. This agrees with the result from previous reports where the investigators showed other pregnancy associated proteins (e.g. PSP60) could be a possible predictor of anomalies in NT recipients (Heyman et al. 2002). The data generated in this study provided new information about hormonal

profile on SCNT-derived pregnancies in dairy cattle and how their perturbation could be related to morphological anomalies of placenta. We also speculated that the measurement of specific hormones such as E2 could be used as a trustworthy tool along with ultrasonography monitoring to predict the critical time when the lives of mother and fetus are threatened.

We assessed some physiological and anatomical anomalies of SCNT placenta that could be used as predictors for compromised pregnancies. We also provided histopathological evidence to reveal how gross anomalies in placenta might be characterized from a histological point of view. In the last phase of the study, we were interested to assess the SCNT placenta at molecular level with the aim to find an explanation to our histopathological observations. To choose our target molecules we reviewed our results. We had chosen E2 as our candidate for monitoring of SCNT gestations, as E2 concentrations were higher in cloned recipients throughout pregnancy. It has been shown that E2 modulates several cell functions, including migration and proliferation (Johns et al. 1996). This action is made possible by effects on the intercellular adhesion through the disconnection of adhesion junction complexes from the cytoskeleton, evoked by a membrane-associated signalling pathway (Groten et al. 2005). The same authors reported the disrupted placental epithelium characterized by local disappearance of epithelial layer specifically in cotyledon villi. Therefore, in the third series of experiments we investigated the epithelial cell adherens junction structure as a mechanism which regulates cell-cell connection in SCNT placentomes. We hypothesized that the alteration in adherens junction structural molecule expression leads to perturbation in essential cell

signalling and cell-cell adhesions involved in placentation events that are important contribution in establishment and continuation of healthy pregnancies. The objective of the study was to evaluate the expression of two main adherens junction structural proteins, E-cadherin and β-catenin and their regulatory role during placentation. We used techniques such as immunohistochemistry, Western blotting and quantitative RT-PCR (qRT-PCR) to achieve this objective. By immunoblotting, we demonstrated reduced expression of E-cadherin and total β-catenin protein in trophectoderm epithelial cells during the window of placentation (Day 40) in SCNT derived embryos. To determine whether the changes in protein expressions are due to the *in utero* environment, we analyzed bovine trophectoderm cell lines from both groups (SCNT and control) in parallel to *in vivo* samples. The sub-cellular localization pattern of target proteins revealed that the membrane distribution of both E-cadherin and β-catenin was reduced in Day 40 SCNT placental trophoblast cells compared to trophoblast cells in normal gestations. We speculated that such a situation could weaken the structure and integrity of trophoblast cells, result in loss of attachment and compromise the nutrient support of the embryo. Also, Western blot results showed no significant differences in E-cadherin and total β-catenin protein expressions between the two groups of trophectoderm cell lines. This suggests that the under expressions of both proteins in SCNT cotyledons is the consequence of miscommunication between fetus and mother in SCNT gestations and not a direct result of the nuclear transfer technique on fetus.

Similar observation have been made on the events in human placenta in cases of spontaneous abortions (Yurdakan et al. 2008) and human gestational trophoblastic diseases (Li et al. 2003). As the reduction in β-catenin expression in SCNT placenta may represent

the alteration in its transcriptional activity via the WNT/β-catenin signalling pathway, we assessed the expression of some of the WNT/β-catenin signal target genes in SCNT placenta. Using qRT-PCR, we identified a significant reduction in the abundance of transcripts for *CCND1, CLDN1* and *MSX1* in placentas from SCNT compared to AI gestations. *MSX1* is believed to regulate the switching from differentiated to undifferentiated state in myotubes (Odelberg et al. 2000). It is also correlated to pre-implantation embryo death in cattle (El-Sayed et al. 2006). Cyclin D1 (*CCND1*) is also believed to be involved in the endoreduplication process in binucleate cells (Nakano et al. 2005). Also, *CLDN1* modulates cellular resistance to apoptosis (Akasaka et al. 2010), cell migration, anoikis and invasion (Dhawan et al. 2005). Considering the critical roles of these target genes in regulation of trophoblast cell formation and function, it is reasonable to speculate that the aberrant expression of E-cadherin and β-catenin proteins in SCNT placenta could lead to the disintegration of placental epithelium that might contribute to detachment of cotyledons, as we have observed in the first series of experiments. These changes eventually compromises fetal wellbeing through perturbation in placental function. However, further studies will be required to investigate the possible post-transcriptional and/or post-translational regulatory mechanisms and specific effects of excessive concentrations of E2 that influence E-cadherin and β-catenin expression in trophoblast cells during SCNT gestation. These studies are expected to provide further insight into the underlying events that control adherence and attachment of trophectoderm during placentation.

General Conclusion and Future perspective

1- In this dissertation, I characterized, for the first time, the morphological and functional (hormone synthesis) anomalies associated with placental development during second and third trimesters of gestation in SCNT pregnancies. It was inferred that hormonal changes, along with morphological anomalies of placenta, result in compromised fetal development. It was also concluded that the placental abnormalities follow a pattern that could be used to predict critical time when the medical intervention can be applied to prevent gestational loss. In addition, it was concluded that ultrasonographic monitoring of pregnancies enabled characterization of changes in the placenta and may be useful to assess fetal well-being.

2- It is shown herein that there are critical stages during SCNT gestation which have varying impacts on survival rate of cloned fetus. In these studies, it was emphasized that the period between Day 150 and Day 180 of pregnancy is a critical time window when the fetal or mother life is threatened. The results demonstrate that problem is preventable by early diagnosis and early medical intervention. These studies will improve the overall efficiency of cloning in cattle

3- The studies in this thesis demonstrated that E-cadherin and β-catenin play a critical role in bovine trophectoderm formation and function. Defective activation of β-catenin during placentation (Day 40) in SCNT-derived fetus could compromise the

feto-maternal attachment and exchange thereby threatening embryo and fetal survival resulting in pregnancy loss.

Future Perspective

Together the results of these experiments represent a significant contribution to the field of bovine SCNT. Our clinical findings are of special interest as they help us to plan preventive strategies with the aim to improve the performance of assisted reproductive techniques such as somatic nuclear transfer. Also, in light of the present results, we gained insight into the roles of adherens junctions during the development of placental abnormalities that might account for some of the inefficiencies associated with SCNT gestations. However, research is needed to develop a deeper understanding of the placentation in nuclear transfer produced animals in order to elucidate the regulatory mechanisms involved with the aim to make SCNT a more efficient process.

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